

FLOODING, SOIL TEMPERATURE, IRON NUTRITION, PHYSIOLOGY, AND
GROWTH OF *Annona* SPECIES

By

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I dedicate this work to my beloved family

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FLOODING, SOIL TEMPERATURE, IRON NUTRITION, PHYSIOLOGY,
AND GROWTH OF *Annona* SPECIES

By

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A study was conducted to evaluate the effects of flooding, soil temperature, and Fe deficiency on physiology and growth of two *Annona* species with potential as flood-tolerant rootstocks, pond apple (*Annona glabra* L.) and soursop (*A. muricata* L.) in comparison with commercially grown 'Gefner' atemoya (*A. squamosa* L. x *A. cherimola* Mill) on sugar apple (*A. squamosa* L.) rootstock. Plants were exposed to soil temperatures of 5, 10, 20, 25 or 35°C in a glasshouse. Soil temperatures at 5 and 10°C decreased net CO₂ assimilation (*A*) and growth of all species. The highest *A*, leaf chlorophyll index, and growth occurred at 20 to 25°C for pond apple, and 25 to 35°C for soursop and 'Gefner' atemoya on sugar apple rootstock. The effect of floodwater temperatures on physiology and growth of pond apple and soursop was also studied using the same soil temperatures. Both species developed hypertrophied trunk lenticels and adventitious roots when flooded at 20, 25, and 35°C. Plants of both species died at 5 and

10°C by 4 weeks after flooding. Flooding increased root electrolyte leakage and reduced leaf chlorophyll index, *A*, and growth of soursop. Pond apple was more flood tolerant than soursop showing the greatest growth at 20 to 25°C soil temperatures.

Pond apple plants grown in calcareous soil were fertilized with 5 different rates of chelated or non-chelated Fe, under flooded or non-flooded conditions. Flooding decreased leaf concentrations of N, P, K, Ca, Mg, Zn, and Cu, and increased Fe and Mn. Plants in chelated, non-flooded treatments had the highest leaf chlorophyll index and growth at 2.5 to 5 g Fe/plant. In general flooded plants supplied with chelated or non-chelated iron had similar leaf chlorophyll indices and growth.

In two different experiments pond apple and soursop were screened for Fe deficiency tolerance in a hydroponic solution containing 0 or 90 µM Fe, or 2, 22.5 or 45 µM Fe. Root ferric chelate reductase (FCR) activity was not induced by either the 0 or 2 µM Fe concentration in both species. Soursop exhibited higher leaf FCR activity than pond apple. Leaf chlorophyll index and growth were severely reduced in pond apple at 0 or 2 µM Fe.

Pond apple is more flood tolerant but less tolerant to Fe deficiency than soursop. Soursop may be a good flood-tolerant rootstock only for areas subjected to short-term flooding. Soursop and sugar apple require higher soil temperatures (25 to 35°C) than pond apple (20 to 25°C) for optimum growth.

CHAPTER 1 INTRODUCTION

Southern Florida is the major tropical fruit production area in the continental United States. The most common environmental stress affecting tropical fruit trees in southern Florida is flooding (Terry, 1993; Schaffer, 1998). Many tropical fruit orchards in Miami-Dade County are located in areas with poorly drained soils or areas prone to cyclical flooding. Recent efforts to revitalize the flora and fauna of the Everglades National Park have increased water flow, resulting in an elevated water table. This practice has already resulted in periodic waterlogging of many agricultural areas (Crane et al., 1997; Schaffer, 1998). Additionally, little documentation exists concerning the extent of flood damage to commercially grown crops in the agricultural areas of Miami-Dade County (Crane et al., 1997).

In general, little information is available concerning sensitivity of tropical fruit crops to flooding (Iles, 1994). An understanding of the effects of waterlogging on the physiology and growth of subtropical and tropical fruit crops may aid in selection of cultivars adapted to flood-prone or poorly drained soils, or suggest cultural methods to improve flood tolerance and increase the agricultural potential of flood-prone areas with limited negative impact on the environment. Some studies in south Florida have focused on determining the effects of continuous and cyclical flooding on growth of the major subtropical and tropical fruit tree species grown in the area (Schaffer, 1998).

The genus *Annona* includes several subtropical and tropical fruit crop species. The most widely cultivated species include cherimoya (*A. cherimola* Mill.), soursop

(*A. muricata* L.), sugar apple (*A. squamosa* L.), atemoya (*A. squamosa* L. x *A. cherimola* Mill.), and bullock's heart (*A. reticulata* L.). Two species of *Annona*, sugar apple and atemoya, are among the 10 tropical fruit crops with the greatest potential for economic success in south Florida (Mazak and Degner, 1994). However, in some areas, annonas are grown in soils that may be affected by continuous or cyclical flooding (Schaffer, 1998).

In a recent study, flood tolerance of flood-sensitive *Annona* species was achieved by grafting several species onto pond apple rootstock (*A. glabra* L.), a flood-tolerant *Annona* species (Núñez-Elisea et al., 1998, 1999). Pond apple is not grown commercially although its fruits have been used to make jams and jellies (B. Schaffer, personal communication). In glasshouse studies, trees grafted onto pond apple and soursop seedling trees were flood tolerant. These trees actually grew better in continuously flooded than in non-flooded conditions for up to 6 months (Núñez-Elisea et al., 1998). However, pond apple is susceptible to Fe deficiency under non-flooded conditions and soursop is susceptible to low temperature damage that may restrict the use of these *Annona* species as flood-tolerant rootstocks in the calcareous soils such as those in southern Florida.

Iron deficiency is common in calcareous limestone soils, which leads to Fe chlorosis of many fruit crops. Iron application to these soils can be a major production cost (Schaffer et al., 1988). Therefore, the development of screening procedures for potential Fe deficiency in fruit crops may result in an economical means to reduce growth and yield losses due to Fe deficiency (Jolley et al., 1996; Castle and Manthey, 1998). Field trials have indicated that in soil with a high pH, scions of commercial *Annona* species grafted onto pond apple rootstock require considerably more Fe than the same

scions on traditional annona rootstocks such as sugar apple and bullock's heart unless the soil is periodically flooded (B. Schaffer, personal communication). Thus, determining the Fe requirement of pond apple rootstock in flooded and non-flooded soils may provide insight into the mechanism of Fe uptake and provide information for more efficient use of agricultural areas subjected to periodic waterlogging.

Preliminary studies have indicated that soursop may have potential as a flood-tolerant annona rootstock (Núñez-Elisea et al., 1999). However, soursop is less tolerant to low air temperatures than other *Annona* species (Campbell et al., 1977). None of the literature addresses the effects of soil temperature on root function of soursop. To assess the potential usefulness of soursop as a flood-tolerant rootstock for *Annona* species in cooler subtropical areas such as south Florida, the ability of this species to withstand low soil temperatures needs to be assessed.

The focus of this research was to evaluate annona responses to environmental stresses such as temperature, flooding, and low Fe concentration. The ultimate objective of this study was to provide information and possible methods for selecting improved rootstocks for annonas for flood-prone areas, particularly where calcareous soils are prevalent such as in southern Florida. Specific goals were:

- To determine the effect of soil temperature on growth and physiology of pond apple, soursop, and 'Gefner' atemoya on sugar apple rootstock.
- To evaluate the effect of floodwater temperature on growth and physiology of pond apple and soursop.
- To determine the optimum Fe level and type of Fe source for pond apple under flooded and non-flooded conditions.
- To screen pond apple and soursop, the two *Annona* species with potential as rootstocks, for Fe-deficiency tolerance in nutrient solutions.

CHAPTER 2 LITERATURE REVIEW

***Annona* Characteristics**

The *Annonaceae* includes several subtropical and tropical fruit crop species. The most important species of economic importance throughout the world are cherimoya (*A. cherimola* Mill.), soursop (*A. muricata* L.), sugar apple (*A. squamosa* L.), atemoya (*A. squamosa* L. x *A. cherimola* Mill.), and bullock's heart (*A. reticulata* L.) (Morton, 1987; Nakasone and Paull, 1998). Cherimoya generally produces the finest quality fruit of all *Annonas* (Popenoe, 1974). In subtropical areas of south Florida, sugar apple and atemoya are grown commercially. The sugar apple is the most widely planted of the two species because it is adapted to south Florida growing conditions, and produces high quality fruit.

Recently, some *Annona* species have also been studied for their medicinal value. Research on soursop (Gleye et al., 1997) and pond apple (Liu et al., 1999) have led to isolation of a great number of compounds that are antiparasitic, pesticidal, antitumoral, and cytotoxic especially related to breast and prostate cancer cell lines.

Annona fruit production varies seasonally and is affected by many environmental factors (George and Nissen, 1992). Soursop is the most tropical among the commercial *Annona* species and therefore is generally not grown commercially in the marine subtropical climate of south Florida (Morton, 1987). Temperatures from 4 to 7°C cause leaf damage and fruit drop of soursop (Popenoe, 1974). Campbell et al. (1977) rated low temperature injury to *Annona* species in south Florida and observed that soursop was

much more sensitive to low temperatures than sugar apple and atemoya. However, soursop is tolerant to flooding (Núñez-Elisea et al., 1999).

Atemoya (also called custard apple in some areas) is a semi-deciduous subtropical species (George et al., 1987). It has shown considerable seasonal variation in fruit production among and within orchards in the same region. Environmental factors, especially relative humidity have been suggested as possible causes for the seasonal variation in atemoya fruit production (George et al., 1990; George and Nissen, 1992). Temperature requirements of atemoya are more tropical than those of cherimoya (Nakasone and Paull, 1998).

Cherimoya originated in the highlands of Peru and Ecuador. It grows naturally at elevations between 200 and 1400 m where the annual mean temperature range between 17 and 20°C (Morton, 1987). Cherimoya is cultivated in coastal regions of California. In Japan, it is grown in warm areas and greenhouses are used for frost protection (Higuchi et al., 1999). About 1,200 ha of cherimoya are grown in Chile for both the internal and export market (Gardiazabal and Cano, 1999).

Sugar apple is the most widely distributed of the *Annona* species. It is less tolerant to low temperatures than cherimoya but more tolerant than soursop (Nakasone and Paull, 1998). Sugar apple is very sensitive to flooding (Núñez-Elisea et al., 1999).

Pond apple is primarily a non-commercial species native to tropical and subtropical wetlands of the Americas, including swamps of south Florida. It is extremely flood tolerant (Zotz et al., 1997; Núñez-Elisea et al., 1999). The use of pond apple as a rootstock may allow production of commercially flood-sensitive *Annona* species (Núñez-Elisea et al., 1998) in flood-prone or poorly drained areas.

Environmental Factors Affecting Growth and Development of *Annona* Species

Temperature

Temperature is one of the primary environmental factors limiting crop production worldwide. Typically, subtropical and tropical crops are susceptible to chilling injury (Khairi and Hall, 1976; George and Nissen, 1992; Whiley et al., 1999). Exposure to air temperatures below 10°C may cause irreversible chilling injury. Chilling can produce a general breakdown of cellular components such as the loss of semi-permeability of the cell membranes (Björkman et al., 1980) including chloroplast membranes in many plant species (Bowers, 1994), and subsequently leading to decreased net CO₂ assimilation (*A*). The chloroplast membranes of all higher plants contain trienoic fatty acids, which ensure the maintenance of chloroplast structure when plants are exposed to low temperatures (<10°C). Chilling sensitive plants have a low proportion of these fatty acids (Routaboul et al., 2000). Chilling can also inhibit *A* directly (Taylor and Rowley, 1971; Whiley et al., 1999). If the chilling occurs in the presence of light (Bowers, 1994), there can be photoinhibitory damage to photosystem 2 (PSII) (Powless, 1984; Barth and Krause, 1999). Photoinhibition damage may be determined by measuring chlorophyll fluorescence (F_v/F_m), which is a decrease in the ratio of the variable fluorescence to maximum fluorescence recorded.

Suboptimal soil temperatures can also cause changes in some physiological process in the root and thus on the whole plant. Plant respiration and carbohydrate metabolism are altered because root sink strength is reduced as consequence a slow root growth caused by suboptimal soil temperature (Delucia, 1986). The expression of genes that code for the synthesis of ribulose biphosphate carboxylase (Rubisco) may also be inhibited as consequence of changes in root sink strength for photoassimilates (Drake et al., 1997;

Koch, 1996). This response may be due to a feedback inhibition from the source (leaves) as a result of reduced sink strength (slow root growth) (Delucia, 1986). Additionally, a reduced synthesis of certain phytohormones such as cytokinins caused by suboptimal soil temperatures (Tromp and Ovaa, 1994) can result in a poor plant growth. Cytokinins promote meristematic growth (cell division) and are also involved in promoting chloroplast development (Chory et al., 1994), and net CO₂ assimilation. Reduced growth caused by suboptimal soil temperature is also associated with limited water uptake and reduced plant hydraulic conductivity. The viscosity of water decreases at low temperatures, which has been reported in many crops (Markhart et al., 1979; Syvertsen et al., 1983; Wilcox et al., 1983). Reduced water uptake leads to reduced ion uptake (Markhart et al., 1979; Welkie, 1995) and thus, to poor plant growth.

Similarly, subtropical species exposed to supraoptimal temperatures may exhibit a general breakdown of cellular components such as the loss of cell membrane fluidity (Björkman et al., 1980). These crops usually lack the thermo-tolerance of tropical species. Some studies have reported that heat shock proteins (HSP) 70s are involved in thermo-tolerance (Sung et al., 2001). These HSP70s are a specific set of HSPs that are induced by a rapid increase in temperature. They can prevent aggregation of denatured proteins under supraoptimal temperature conditions.

Responses of *Annona* Species

Many subtropical annona production areas, such as south Florida, are subject to low temperatures. The effect of low air temperature on annonas is more pronounced for soursop than for sugar apple, pond apple, or atemoya. Branches of juvenile soursop plants were killed when air temperatures were below 0°C (Campbell et al., 1977). George and

Nissen (1987) reported increased dry matter accumulation at root temperatures of 15°C than at 28°C for most cherimoya cultivars.

Supraoptimal temperature also affects growth and development of annonas. The growth rate of cherimoya is reduced during hot seasons, even in temperate areas. Higuchi et al. (1999) found that leaf chlorophyll content, leaf number and area, and net CO₂ assimilation of cherimoya were all lower at day-night temperatures of 30/25°C than at 20/15°C. In addition, Higuchi et al. (1998a) reported that day-night temperatures of 30/25°C had an adverse effect on pollen germination of cherimoya. In contrast, George and Nissen (1988) observed that growth of atemoya was generally lower at day-night air temperatures of 25/18°C than at 28/23°C. Higuchi et al. (1998b) found similar results for sugar apple using day-night air temperatures of 20/15°C and 30/25°C under sunlit glasshouse conditions. Yamada et al. (1996) also determined that soursop and sugar apple had heat tolerant leaves by measuring chlorophyll fluorescence.

Flooding

Flooding responses of fruit crops are directly related to changes in O₂ availability, and the chemical and physical states of the soil that occur after flooding (Andersen et al., 1984a, 1984b; Crane and Davies, 1989; Larson et al., 1993; Else et al., 1996). Flooding can produce hypoxia (low O₂ concentration) or anoxia (lack of O₂). Plants respond to flooding in many different ways, depending on duration, season, and inherent flood tolerance (Crane and Davies, 1988, 1989; Ranney and Bir, 1994; Núñez-Elisea et al., 1998). Flooding during the growing season typically causes more damage to trees than flooding during dormant periods (Iles, 1994).

Flood-stressed trees exhibit a wide range of symptoms that have profound effects on plant growth and development (Schaffer et al., 1992; Iles, 1994; Schaffer, 1998). The

primary effect of flooding on crops is a reduction in root and shoot growth due to decreased soil oxygen content (Schaffer et al., 1992). Crane and Davies (1989) defined three phases of plant response that occur under flooding conditions for *Vaccinium* species. The exact time required for each phase varies among species and cultivars, and depends on environmental factors, such as air and soil temperature and relative humidity.

Various physiological and metabolic processes are also negatively affected by flooding, including decreases in net CO₂ assimilation (*A*) (Núñez-Elisea et al., 1999; Zude-Sasse et al., 1998; Larson et al., 1991a), stomatal conductance to CO₂ (*g_s*) (Davies and Flore, 1986; Crane and Davies, 1987), transpiration (*E*) (Davies and Flore, 1986; Crane and Davies, 1989), and root hydraulic conductivity (Syvertsen et al., 1983; Crane and Davies, 1987).

Flooding also changes phytohormone levels in plants, and promotes accumulation of abscisic acid (ABA) (Reid and Bradford, 1984; Zhang and Davies, 1987; Castonguay et al., 1993; Zhang and Zhang, 1994; Rijnders et al., 1997) and ethylene (Reid and Bradford, 1984; Larson et al., 1993; Jackson et al., 1994; Pezeshki et al., 1996; Banga et al., 1997). Exactly how flooding promotes ABA accumulation is not clear. Abscisic acid is commonly associated with stomatal closure and senescence (Reid and Bradford, 1984; Zhang and Zhang, 1994), and appears to induce stomatal closure through its effect on potassium regulation of guard cell turgor (Sojka, 1992). Ethylene also plays an important role in plant response to both biotic and abiotic stresses. Accelerated synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) occurs in roots after the induction of ACC synthase genes by hypoxia (Gur et al., 1998; Grinhko and Glick, 2001). Whether or not a target gene will be affected by ethylene depends on the availability of a free

ethylene sensor and a functional signal transduction chain in the cell. If no receptor is available, ethylene either dissolves in the lipids or diffuses out of the tissue (Grichko and Glick, 2001). Ethylene may produce accelerated senescence (Banga et al., 1997), petiole elongation (Rijnders et al., 1997), stem lenticel hypertrophy (Larson et al., 1991b; Larson et al., 1993; Yamamoto et al., 1995), formation of porous aerenchyma (Jackson et al., 1994), and adventitious rooting (Reid and Bradford, 1984; Yamamoto et al., 1995).

Flooding affects cellular processes, and thus reduces the supply of available energy. Results of a number of investigations suggest that short-term exposure of roots to severe hypoxia or anoxia causes loss of their membrane permeability (Buwalda et al., 1988). Alternatively, hypoxia may induce depolarization of the membrane. This depolarization during hypoxia might explain the losses in cations (e.g. K^+ , Ca^{2+} , Mg^{2+} , and basic metabolites) and the concurrent loss of balancing anions (e.g. Cl^- , organic acids and other acidic metabolites) (Buwalda et al., 1988). Crane and Davies (1987) observed that electrolyte leakage from roots of rabbiteye blueberry occurred 6 d after flooding followed by decreases in stomatal and hydraulic conductances.

Flooding detrimentally affects many tropical and subtropical fruit tree species cultivated in Florida, including mango (Larson et al., 1991a, 1991b; Schaffer et al., 1994; Zude-Sasse et al., 1998), citrus (Syvertsen et al., 1983), carambola (Joyner and Schaffer, 1989; Razi and Mohd, 1996), and several species of *Annona* (Núñez-Elisea et al., 1998, 1999).

Responses of *Annona* Species

Effects on growth and development. Flooding of commercial *Annona* species, even for short periods, reduces growth, may cause defoliation, and severely reduces flowering and fruit set (Marler et al., 1994). Commercial fruit crops in the *Annonaceae*

such as cherimoya, sugar apple, and atemoya are considered flooding sensitive (Núñez-Elisea et al., 1998, 1999).

Severe damage to sugar apple seedlings and rootstocks, and bullock's heart rootstocks such as leaf wilting and necrosis, followed by defoliation have occurred by the fourth week of flooding (Núñez-Elisea et al., 1998, 1999). Growth rates of all species decreased after 6 months of flooding, as evidenced by shorter internodes, reduced flushing frequency, and diminished leaf size. Flooded trees of '4-5' (an *Annona* selection by G. Zill of Zill's High Performance Plant Nursery, Boynton Beach, Florida) grafted on bullock's heart rootstock and 'Gefner' atemoya grafted on sugar apple rootstock showed a 60% reduction in shoot growth after 17 d of flooding compared to non-flooded trees of the same scion-rootstock combinations (Núñez-Elisea et al., 1999).

Physiological processes. Núñez-Elisea et al. (1998) reported significant reductions in leaf gas exchange of bullock's heart and sugar apple (50% of non-flooded control) within 24 h of flooding in these sensitive *Annona* species. The effect was greater for trees flooded continuously than for those exposed to cyclical flooding (Núñez-Elisea et al., 1999).

Flooding tolerance and morphological adaptations of *Annona*. Plant tolerance to flooded soil conditions may be influenced by many factors including soil type, degree and duration of anaerobiosis, pathogen status, vapor pressure deficits, root and air temperatures, plant age, and stage of development (Schaffer et al., 1992). Some plants can overcome flooding stress by either avoiding oxygen deficits (i.e., by developing morphological adaptations such as formation of aerenchyma or hypertrophied stem lenticels for improved root gas exchange and excretion of potentially toxic metabolites),

or by developing physiological adaptations to oxygen deficits (Crawford and Braendle, 1996). These adaptive responses to oxygen deficits include changes in the enzyme profile in roots such as alcohol dehydrogenase (ADH) (Sachs et al., 1996), preservation of membrane integrity (Crawford and Braendle, 1996), regulation of cytoplasm acidosis (Davies et al., 1974; Roberts et al., 1985; Pezeshki et al., 1996; Tamura et al., 1996a), and use of an alternative respiratory pathway (Tamura et al., 1996b; Zude-Sasse et al., 1998). These alternative pathways act as an over-flow for accumulating reduced pyridine nucleotides and plants can further metabolize pyruvate by fermentative metabolism and thus produce cellular energy under limited oxygen conditions.

Núñez-Elisea et al. (1998, 1999) found that selected species of *Annona* trees grafted onto pond apple rootstock, and seedlings of soursop were very flood tolerant. Pond apple and soursop seedlings, and '49-11' (an *Annona* selection by G. Zill of Zill's High Performance Plant Nursery, Boynton Beach, Florida) grafted onto pond apple survived 12 months of continuous flooding. Pond apple, either as a seedling tree or rootstock, developed adventitious roots, hypertrophied (swollen) lenticels on the trunk, adventitious roots, and basal trunk swelling (Núñez-Elisea et al., 1998). Soursop seedlings exposed to flooded conditions produced vegetative shoots at the base of the trunk, but no trunk swelling or hypertrophied lenticels were observed. Differences in morphological adaptations to flooding between pond apple and soursop suggest that each species may possess different physiological mechanisms and alternate metabolic pathways for increasing oxygen uptake and eliminating potentially toxic metabolic byproducts of anaerobic respiration in the roots.

Zotz et al. (1997) reported that pond apple has an efficient hydraulic system. More than two thirds of whole plant resistance to water flow in pond apple was attributed to the roots. Low hydraulic conductivity of roots was compensated for by an increase in root number.

Effects on soil chemistry, redox potential, and iron availability

The redox potential (Eh) is a quantitative measurement of the electrochemical status of the soil redox system, and its measurement serves as an indicator of soil oxidation/reduction status under flooded conditions (Patrick and DeLaune, 1977; Schaffer et al., 1992). Flooding alters the oxidation-reduction (redox) status of the soil by reducing the redox potential due to chemical changes and production of byproducts by soil microbes (Ponnamperuma, 1984). Redox potentials ≥ 300 mV are indicative of aerobic conditions, whereas potentials ≤ 200 mV suggest anaerobic conditions. Therefore, soil Eh may be a useful indicator of plant survival in a given soil type. However, plant species differ in their ability to withstand certain levels of reduced soil conditions depending on the intensity of reduction of chemical elements (Pezeshki, 1991; Pezeshki and DeLaune, 1998). The rate and degree of soil reduction is also dependent on soil pH, temperature, organic matter content, and concentration of alternative electron acceptors. Redox potential might be the most significant single factor affecting Fe transformation in flooded or periodic flooded soils (Sah et al., 1989). The critical redox potential at which Fe^{3+} is reduced to Fe^{2+} is 100 mV (Patrick and Jugsujinda, 1992).

Núñez-Elisea et al. (1999) reported that flooding reduced the Eh of Krome very gravelly loam soil (loamy-skeletal, carbonatic, hyperthermic Lithic Rendoll), from 423 mV to 196 mV, indicating that the soil was anaerobic after 3 d, and Eh

decreased gradually until day 15 after plants were flooded. Average Eh values changed little after 20 d of flooding remaining at about -155 mV until the end of the study (day 50). Syvertsen et al. (1983), Crane and Davies (1988) and Larson et al. (1991b) observed similar results in other soils for citrus, rabbiteye blueberry, and mango, respectively.

In alkaline soils, flooding decreases soil pH (Larson et al., 1991c). Under flooded conditions, nutrient elements such as Fe, Mg, and Mn to become more soluble (Ponnamperuma, 1984; Larson et al., 1991c). Iron in the form of Fe^{3+} is reduced to Fe^{2+} , which is more available to plants (Ponnamperuma, 1984; Larson et al., 1991c, 1992; De Mello et al., 1998; Zude-Sasse and Lüdders, 2000; Zude-Sasse and Schaffer, 2000). The nature and magnitude of Fe transformations in periodically flooded soils depend on interactions among flooding period, organic matter, and temperature (Sah et al., 1989).

The reduction of Fe and its increased solubility is often one of the most important chemical changes that occurs in flooded soils. Short-term soil anoxia may enhance Fe uptake (Laan et al., 1989; Zude-Sasse and Lüdders, 2000; Zude-Sasse and Schaffer, 2000). This was also reported in two native south Florida soils, Chekika and Krome very gravelly loam. Larson et al. (1991c, 1992) reported that in these calcareous soils increased solubility of Fe occurred after 7 weeks of continuous flooding compared to non-flooded conditions. Pond apple growing in non-flooded calcareous soils in south Florida exhibits symptoms of Fe deficiency (B. Schaffer, personal communication). Thus, periodic flooding in calcareous soils may result in increased micronutrient availability and improved plant nutritional status. Although the use of chelated Fe fertilizers is required in the alkaline agricultural soils, the periodic flooding that occurs in the natural

habitat of pond apple presumably results in a higher availability of Fe by an increased Fe^{2+} solubility (B. Schaffer, personal communication).

The effects of flooding on nutrient absorption differ between flood-intolerant and flood-tolerant species. Nutrient absorption actually may increase in flood-tolerant plants under flooded conditions, whereas flooding decreases the absorption of mineral nutrients in flood-intolerant species (Schaffer et al., 1992). Reduced nutrient uptake under hypoxic conditions could be attributed to several factors, including root mortality, reductions in root respiration, and root hydraulic conductivity.

High-pH Soil and Iron Availability

A common problem in calcareous soils is an inadequate supply of available Fe to the plant. Calcareous soils have high pH (7.5 to 8.5), high levels of bicarbonate (HCO_3^-), and generally a low organic matter content (Lucena, 2000). These soil conditions lead to Fe deficiency chlorosis in many fruit crops (Korcak, 1987; Tagliavini and Rombolá, 2001). Bicarbonate is the most important soil factor associated with lime-induced chlorosis. It is a strong pH buffer, mainly in the presence of calcium carbonate, which prevents root apoplast acidification due to consumption of H^+ extruded by the proton ATPase and inhibition of Fe^{3+} reduction by root cells (Toulon et al., 1992; Shi et al., 1993; Marschner and Römhild, 1995; Mengel, 1994; Lucena, 2000; Nikolic et al., 2000; Alcántara et al., 2000). Iron deficiency is caused more by bicarbonate concentration than by high soil pH (Romera et al., 1991a). In addition, calcareous soils tend to accumulate more nitrate than ammonium because at high pH ammonium nitrogen is rapidly nitrified and may volatilize as NH_3 (Mengel et al., 1994). These soil conditions decrease growth and yields, cause leaf chlorosis, and changes in the root morphology (Welkie, 1995; Zouari et al., 2001; Rosenfield et al., 1991). Yellowing of new leaves is evidence for Fe

chlorosis. Symptoms of Fe deficiency first appear on younger leaves as interveinal chlorosis, sometimes with necrotic spots on the leaves, followed by a reduction of leaf size (Thomas et al., 1995). Leaves may recover during the growing season, but in general the yield is reduced.

Since fruit tree crops are perennial, Fe deficiency in one year may affect subsequent Fe nutrition and chlorosis development in the next year. Additionally, fruit trees also have a much higher shoot/root ratio than annual plants and therefore Fe uptake per unit of root area might be insufficient to fulfill the Fe requirement. The critical concentrations of Fe in leaves required for optimal growth vary widely among species; the usual range is between 50 and 150 mg·kg⁻¹ dry weight (Marschner, 1995). Thus, the ability to identify and correct mineral nutrient deficiencies before they affect tree vigor and limit yields is crucial for optimizing crop production.

Iron has a direct physiological role in processes such as photosynthesis, chlorophyll synthesis (ALA, δ -aminolevulinic acid), respiration, nitrogen fixation, and enzyme activity (Abadía, 1992; Marschner, 1995; Terry and Zayed, 1995). Almost all of a plant's iron is located in the chloroplasts. The yellowing of leaves occurs because the plant cannot maintain its chlorophyll content (Miller et al., 1995). Iron is also a constituent of many electron carriers in the electron transport chain.

The ferric form (Fe^{3+}) must be reduced before entering the root cell (Chaney et al., 1972; Lucena, 2000). Iron transport across the plasma membrane is initiated by Fe^{3+} reduction, which is catalyzed by a root plasma membrane-bound enzyme, ferric chelate reductase (FCR) (Marschner and Römheld, 1995). Chemical reduction of Fe^{3+} depends on the pH. The lower the pH, the more favored the formation Fe^{2+} than Fe^{3+} in the

rhizosphere (Lucena, 2000). Thus, although Fe is one of the major soil constituents (0.5-5%), it is usually present in the oxidized state (Fe^{3+}) so, plant availability is severely limited by the low solubility of Fe at pH levels favorable for plant growth. Many other factors can induce low Fe uptake, among them: insufficient Fe in the media, bicarbonate buffering, nitrate effects on pH and redox state, presence of other metals that can alter the Fe uptake mechanism, inefficient carriers of Fe within the roots, and other soil factors such as low temperature, and CO_2 and ethylene concentrations (Lucena, 2000).

Iron distribution within the plant may be inefficient due to changes in the pH of the sap (bicarbonate and nitrate), or increases in organic acid concentration, and K^+ (Lucena, 2000). Another important factor for plant Fe nutrition is the transport of Fe to the shoots. Fe^{3+} is transported mostly as a citrate complex (Chaney, 1989). Iron(III)-citrate also needs to be reduced by a leaf plasma membrane Fe reductase (FCR) to enter the cell as Fe^{2+} (Brüggemann et al., 1993; Mengel et al., 1994; De la Guardia and Alcántara, 1996; Nikolic and Römheld, 1999). Iron availability for reduction in the leaf apoplast is a second important step necessary before Fe can be available for leaf metabolism. Therefore the pH of the leaf apoplast is also important in controlling availability of Fe (Yu et al., 2000).

Iron uptake is regulated by the whole plant, shoot, or root. Therefore, plants need special mechanisms for acquiring Fe from soluble Fe forms to fulfill growth requirements, especially in neutral and alkaline soils (Neumann and Römheld, 2001). The usual solution to overcoming the unavailability of Fe is either not to plant in such soils or to use soil-applied chelates, or foliar Fe applications. Corrective measures such as application of Fe chelates or sulfuric acid to the soil, and foliar sprays are expensive and

in some cases give inconsistent results or are not feasible (Cinelli et al., 1995). Genetic advances in resistance to Fe deficiency have been impeded by inconsistent symptom expression. Soil heterogeneity and environment are responsible for these inconsistent results. Field trials are expensive, time consuming, and sometimes difficult to interpret.

Another way of overcoming Fe deficiency is to use Fe-deficiency tolerant rootstocks (Castle and Manthey, 1998). A better understanding of the mechanisms involved in overcoming Fe deficiency could lead to the development of a more efficient screening technique to select Fe-deficiency tolerant rootstocks. The development of improved screening techniques for Fe deficiency is the most economically feasible solution to the chlorosis problem (Jolley et al., 1996; Castle and Manthey, 1998). Quantification of Fe reduction by roots and/or H^+ ion extrusion in dicots and monocots (except grasses), or secretion of non-proteinaceous amino acids called phytosiderophores by grasses show potential for screening a wide range of species (Mengel et al., 1994; Marschner and Römheld, 1995; Jolley et al., 1996). The Fe^{3+} reducing capacity by roots is enhanced by Fe deficiency. This is consistent with the increase of subsequent root uptake and translocation rates of Fe in dicots and monocots, except grasses (Nikolic et al., 2000). Bicarbonate, Fe, and other metals affect this response (Alcántara et al., 2000).

Two different types of root response to Fe deficiency (strategies) have been identified: Strategy I and Strategy II (Marschner, 1995; Marschner and Römheld, 1995). Strategy I plants (dicots and monocots, except grasses) are characterized by increasing a plasma membrane-bound inducible reductase activity, an enhanced net excretion of protons, and/or in many cases enhanced release of reductants/chelators (mainly phenolics). The relative importance of the three components seems to differ considerably

among plant species (Marschner and Römheld, 1995). These physiological responses are turned off once acceptable levels of Fe have been obtained (Jolley et al., 1996). When Fe availability limits growth of plants that use Strategy I, changes in root morphology, such as the formation of root hairs may be induced (Schmidt, 1999). In addition, several biochemical responses are induced that increase Fe uptake (Marschner and Römheld, 1995; Jolley et al., 1996). Plants that use Strategy I are called “Fe-deficiency tolerant genotypes”. Dicots and monocots, except grasses, that do not manifest any physiological responses induced by Fe deficiency are called “Fe-deficiency susceptible genotypes.” Characteristics of Strategy I plants are discussed in the following section.

Iron reduction and Fe chelate reductase enzyme. The obligatory reduction of Fe^{3+} to Fe^{2+} is increased by Fe deficiency (Chaney et al., 1972; Marschner and Römheld, 1995; Castle and Manthey, 1998; Nikolic et al., 2000), and there is a high correlation between Fe reducing capacity and Fe uptake (Jolley et al., 1996; Nikolic et al., 2000). Plasma membrane-bound Fe reductase transfers electrons from cytoplasmatic reduced pyridine dinucleotides to the apoplast (Marschner, 1991, 1995). Either natural or synthetic ferric complexes can act as electron acceptors (Lucena, 2000). In Fe-deficiency tolerant plants, the root ferric chelate reductase (FCR) activity varies between 10 to 92 nmol $\text{Fe g}^{-1}\text{FW}\cdot\text{min}^{-1}$ (Moog and Brüggemann, 1994). This FCR transfers electrons from cytoplasmatic reduced pyridine dinucleotides to the apoplast. Higher reduction rates occur with NADH than with NADPH as an electron donor (Moog and Brüggemann, 1994; Marschner and Römheld, 1995). In citrus, some studies have suggested that NADH significantly increased the rates of Fe^{3+} reduction by lignin (Manthey, 1992). Transport of the reduced Fe^{2+} across the plasma membrane is presumably mediated by ion-specific

channels or carriers (Marschner and Römheld, 1995). The pH optimum for Fe reduction ranges from 6.5 to 7.0, but is typically 6.8 (Moog and Brüggemann, 1994).

Acidification of the rhizosphere. Roots of Strategy I plants can also respond to low Fe availability by lowering the rhizosphere pH. ATPases are induced, which pump H^+ ions out of root cells. This ATPase-driven proton pump lowers the pH of both the root apoplast and the rhizosphere. This enhanced acidification significantly increases the solubility of Fe^{3+} compounds in both the root apoplast and the soil solution, and Fe uptake is enhanced (Jolley et al., 1996; Neumann and Römheld, 2001). Nitrate nutrition results in less rhizosphere acidification than ammonium nutrition (Marschner, 1995) because nitrate induces a net release of OH^- by roots and an accumulation of organic anions in the plant (Mengel and Geurtzen, 1988).

Some research has reported that the Fe-chlorosis tolerance is more closely related to Fe reduction than H^+ release (Alcántara et al., 1991). However, Wei et al. (1997) found that H^+ release correlated with chlorosis scores, and proved to be a better predictor of Fe deficiency resistance in the field than root Fe reduction. These observations suggest that each species may have to be tested individually to determine the physiological factor, which is the best for screening resistance to Fe deficiency. The system is very complex. Perhaps quantification of both H^+ release and Fe^{3+} reduction are better selection criteria than measurement of either factor alone (Jolley et al., 1996).

Release of reductants/chelators. Another response of Strategy I plants to Fe deficiency is to release reductants or chelators. Over 99% of the organic acids lost by the root remain within 1 mm of the root surface. Some organic acids, such as citrate, can become highly negatively charged over a wide range of soil conditions allowing them to

react strongly with both the aqueous and solid phases of the soil (De Vos et al., 1986; Jones et al., 1996; Jolley et al., 1996). The accumulation of organic acids in the root tissue in response to Fe limitation not only provides protons for the H^+ -ATPases-mediated rhizosphere acidification but also provides electrons for the Fe deficiency-induced plasma membrane-bound reductase system (Neumann and Römheld, 2001). At pH values ≤ 6.8 , citrate forms stable complexes with Fe and dissolution of Fe proceeds rapidly. Application of Fe(II)-humic complexes has produced a significant increase in Fe assimilation (García-Mina et al., 1995).

Recently, De Nisi and Zocchi (2000) have suggested a role of phosphoenolpyruvate carboxylase (PEPC) as another physiological response to Fe deficiency in calcareous soils. This enzyme incorporates bicarbonate into phosphoenolpyruvate, generating oxalacetate, which is a precursor of citrate and Fe is transported to the shoot as Fe^{3+} -citrate (Tiffin, 1970).

Phytohormones such as ethylene and indoleacetic acid have been implicated in the signaling of the coordinated Strategy I responses to Fe deficiency in dicots (Neumann and Römheld, 2001). Romera et al. (1999) reported that ethylene is involved in the regulation of Fe-deficiency stress responses by Strategy I plants. They found that roots from Fe-deficient plants produce more ethylene than those of Fe-sufficient plants. The higher production of ethylene in Fe-deficient plants occurred before they exhibited chlorosis symptoms.

Strategy II plants (grasses) are characterized by increases in the biosynthesis and secretion of non-proteinaceous amino acids that are highly effective as Fe^{3+} chelators. These compounds are called phytosiderophores (i.e., mugineic acid), which allow the

enhanced solubilization of Fe^{3+} and subsequent uptake of the ferric chelate complex or chelation with Fe^{3+} . Uptake of Fe^{3+} -phytosiderophores is mediated by a specific transporter in the plasma membrane of roots cells of grasses (Marschner and Römheld, 1995; Moog and Brüggemann, 1994). In plants with the Strategy II mechanism, release of phytosiderophores is induced by Fe deficiency, and it is a useful parameter for genotypical characterization of differences in Fe-deficiency tolerant plants (Marschner, 1995). Additionally, some investigations suggest that some grasses may have other mechanisms of Fe uptake besides a release of phytosiderophores (Wang and Peverly, 1999). These Fe^{3+} -phytosiderophore chelates are stable even at soil pH levels > 7.0 (Neumann and Römheld, 2001). Thus, Fe mobilization and Fe uptake in Strategy II plants are less dependent on the external (soil) pH than plants using Strategy I, which is very important especially in calcareous soils with high pH (Neumann and Römheld, 2001).

Studies with *Arabidopsis thaliana* (Strategy I plant) have provided information on the molecular mechanism and regulation of Fe uptake. The isolated *IRT1* (iron-regulated-transport) gene is encoding a Fe^{2+} transporter in the roots and, is induced by Fe deficiency. *IRT1*-mediated uptake of Fe^{2+} was temperature and concentration dependent (Eide et al., 1996). Similarly, Robinson et al. (1999) reported that the *FRO2* gene was expressed in Fe-deficient roots of *Arabidopsis*. *FRO2* belongs to a super-family of flavocytochromes that transport electrons across membranes. *FRO2* is allelic to the *frd1* mutations that impair the activity of ferric-chelate reductase.

Studies on the induction of Fe-stress responses have been conducted with many fruit crops, including grape (Nikolic et al., 2000; Dell'Orto et al., 2000), peach (Romera et al., 1991a, 1991b; De la Guardia et al., 1995; Gogorcena et al., 2000; Alcántara et al.,

2000), quince (Tagliavini et al., 1995), citrus (Manthey et al., 1994; Castle and Manthey, 1998; Pestana et al., 2001), pear (Tagliavini et al., 1995), avocado (Manthey and Crowley, 1997), and papaya (Marler et al., 2002).

Studies have shown that FCR activity is localized either in the root apex (Marler et al., 2002) or subapical zone or in secondary roots (Gogorcena et al., 2000). However, some studies have indicated an inconsistent relationship between FCR activity in whole plant and in root tip assay procedures. This may be due to the occurrence of “patchiness” in root FCR activity, with some root tips reducing much more Fe than others tips of the same plants (Gogorcena et al., 2000).

Pestana et al. (2001), studying induction of Fe-stress responses in citrus, found an increase in root FCR activity either in absence of Fe or in low concentration of Fe but only when calcium carbonate was added to the nutrient solution. They suggested that for citrus species a low level of Fe was not sufficient by itself to induce an increase in root FCR activity. Conversely, some studies have reported that a small amount of Fe in the nutrient solution is apparently required to induce a significant induction of the root FCR activity of some Fe deficient plants (Romera et al., 1991b; Tagliavini et al., 1995; Romera et al., 1996; Gogorcena et al., 2000; Zouari et al., 2001). This suggests that when some species are grown in nutrient solutions without Fe, plants continue growing and exhibiting leaf chlorosis but do not exhibit an increase in root FCR activity. Gogorcena et al. (2000) suggested that the addition of Fe might trigger increases in root FCR activity in three ways. First, roots growing with no Fe may not be as healthy as those growing with a small amount of Fe. Second, a complete lack of Fe deficiency would cause low activity of the Fe-dependent enzyme ACC synthase, an enzyme in the ethylene biosynthesis

pathway; ethylene may be somehow required for FCR activity (Romera and Alcántara, 1994; Romera et al., 1996, 1998). Third, an Fe-containing component may be necessary for functioning of FCR itself. For instance, a flavocytochrome has been identified as one of the proteins responsible for FCR activity in *Arabidopsis* (Robinson et al., 1999).

A higher Fe concentration can be found in young chlorotic leaves than in green leaves, which has been called “the chlorosis paradox” (Mengel, 1994; Römheld, 2000). That chlorosis might be caused by Fe inactivation in the plant, in particular in the leaf apoplast, e.g. by an alkalization process (Mengel et al., 1994). However, Römheld (2000) reported that Fe deficiency may impair leaf expansion resulting to a relative increase in Fe concentration in chlorotic leaves.

Leaf chlorophyll concentration can also be used to classify tolerant cultivars to Fe deficiency and should be used in combination with indices of relative growth (De la Guardia et al., 1995). However, other environmental conditions could alter the capacity to absorb Fe from the soil, along with problems linked to the translocation of Fe to the shoot (Dell’Orto et al., 2000).

CHAPTER 3
SOIL TEMPERATURE, PHYSIOLOGY, AND GROWTH OF CONTAINERIZED
Annona SPECIES

Introduction

The *Annonaceae* includes several subtropical and tropical fruit crop species. The species of most economic importance throughout the world include cherimoya (*A. cherimola* Mill.), sugar apple (*A. squamosa* L.), soursop (*A. muricata* L.), and atemoya (*A. squamosa* L. x *A. cherimola* Mill.) (Morton, 1987; Nakasone and Paull, 1998).

Annona fruit production is affected by many environmental factors such as flooding (Crane et al., 1997; Núñez-Elisea et al., 1998, 1999), air and soil temperatures (Campbell et al., 1977; George and Nissen, 1987; Higuchi et al., 1999), and relative humidity (George and Nissen, 1992). Recent studies suggest that soursop and pond apple (*A. glabra* L.) have potential for use as flood-tolerant rootstocks for commercial *Annona* species (Núñez-Elisea et al., 1998, 1999). Soursop is the most tropical of the *Annona* species (Morton, 1987) and grows well under tropical lowland climate conditions. Pond apple is native to tropical and subtropical wetlands of the Americas (Nakasone and Paull, 1998).

The effect of air temperature on growth, development, and physiological processes of some *Annona* species has been extensively studied (George et al., 1990; George and Nissen, 1992; Yamada et al., 1996; Higuchi et al., 1998a, 1998b, 1999). The effect of air temperature on annona growth depends on the species. Cherimoya appears to initiate

growth at 7°C and atemoya at 10°C (George et al., 1987). The effect of low air temperature on annonas is more pronounced for soursop than for sugar apple, pond apple, or atemoya since branches of juvenile soursop plants were killed when air temperatures were below 0°C (Campbell et al., 1977).

There is a general lack of information about the effects of soil temperature on physiology and growth of *Annona* species. Understanding the response of *Annona* species to soil temperature should help in selection of annona rootstocks or seedling trees for specific areas.

The objective of this study was to determine the effect of soil temperature on physiology and growth of selected *Annona* species. Pond apple and soursop seedlings were tested based on their potential as flood-tolerant rootstocks. The soil temperature responses of these two species were compared to those of atemoya on sugar apple rootstock, which is a commonly grown scion species on a traditionally used (George et al., 1987), flood-sensitive annona rootstock (Núñez-Elisea et al., 1999).

Materials and Methods

The study was conducted from March through November 2000 in a sunlit glasshouse at the University of Florida in Gainesville, Fla. Average day/night air temperatures during the experimental period ranged from 40/16 to 28/8°C and relative humidity averaged 80 to 90%.

Plant Material

Seedling trees between 1 and 1.5 years of age were used in this experiment. Trees of soursop (*A. muricata* L.), pond apple (*A. glabra* L.), and ‘Gefner’ atemoya (*A. squamosa* L. x *A. cherimola* Mill.) on sugar apple (*A. squamosa* L.) rootstock were grown in 3.8-L containers in well-drained media containing peat, sawdust, and sand

(1:1:1 by volume). Plants were irrigated every day to container capacity, and fertilized (top dressing) every 5 weeks with 15 g Osmocote Plus Controlled Release® fertilizer (15N-4P-10K, with ammonium and nitrate as the N sources).

Treatments

Six weeks prior to initiating the treatments, all plants were pruned to produce new growth flushes. Uniform trees of each species were selected and placed in each of five root temperature chambers and subjected to one of five soil temperatures (5, 10, 20, 25, or $35 \pm 2^{\circ}\text{C}$). There was one root temperature chamber for each soil temperature treatment and the chambers were used for the same temperatures for each replication. Six containerized trees were placed in each chamber (2 of each species). Due to the limited number of root temperature chambers, treatments were replicated and blocked over 4 times for a total of 4 replications over a 1-year period. The treatments were replicated from March-May, June-August, August-October, and October-November. Plants in each replication were subjected to the treatments for 6 weeks. This approach was previously used for carambola (*Averrhoa carambola* L.) (George et al., 2002)

Root Chambers

The root temperature chambers used for the experiment were thermostatically controlled freezers that were modified by removing the lids and replacing them with styrofoam lids. The Styrofoam lids allowed for potted plants to be positioned with roots in the controlled-temperature chamber and canopy exposed to the ambient air temperature. The top of each plant container was insulated with styrofoam similar to that used for the lids of the root temperature chambers to reduce evaporation from the potting media and moderate temperature changes, while allowing adequate aeration of the roots.

A two-speed oscillating fan was placed inside each of the growth chambers to help maintain uniform temperatures throughout the chamber and to allow for air circulation. Five 20-L plastic buckets filled with water were placed inside the 5, 10, and 20°C chambers to help maintain constant temperatures. For the chambers at 25 and 35°C, the entire chambers were half-filled with water to help maintain constant temperatures. A VISI-THERM aquarium water heater (Aquarium Systems, Mentor, OH) was installed below the water level inside the 35°C chamber to maintain the temperature. The ambient air temperatures in the glasshouse and soil temperatures in each root temperature chamber were continuously monitored and recorded with a Hobo H8 Pro Series temperature logger (Onset Computer Corporation, Pocasset, Mass.) and a Fisher mercury thermometer (Fisher Scientific Co. LLC, Ill). The thermometers monitoring soil temperatures were positioned at the center of the rooting medium in one of the containers in each chamber. Prior to their installation, the temperature loggers were calibrated by comparing temperatures to temperatures obtained with a mercury thermometer in ice-water and in water at ambient temperature. Ambient air temperatures and RH in the glasshouse were measured at a height of 1.82 m above ground level.

Physiological Measurements

Leaf chlorophyll index was determined with a chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd, Japan). Chlorophyll measurements were made on six randomly selected leaves per tree. Leaf chlorophyll index was measured on the same leaves used for gas exchange measurements.

Net CO₂ assimilation (*A*), stomatal conductance for CO₂ (*g_s'*), and transpiration (*E*) were measured weekly using a portable infrared gas analyzer (LCA-2, Analytical Development Co. LTD, England). Measurements were made between 1200 and 1500 HR

at a photosynthetic photon flux (PPF) $>700 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which is above light saturation for annona photosynthesis (Marler and Zozor, 1996; Utsunomiya and Higuchi, 1996), with sunlight as the light source. The most recently matured, fully exposed leaves located at the 4th or 5th node below the shoot apex were selected for gas exchange measurements.

Growth Measurements

Plant height, trunk diameter, axillary shoot growth, the number of leaves per tagged shoot, and fresh and dry weights were measured for all plants. All growth measurements, except trunk diameter and fresh and dry weights, were made at the beginning (day 1) of each replication and repeated weekly for 6 weeks. Trunk diameter was recorded every 2 weeks.

Plant height was determined on the main stem, which was measured from the surface of the planting medium to the apical bud. Trunk diameter was measured at a 10-cm height above the surface of the planting medium using a micrometer caliper. Axillary shoot growth was obtained by measuring the length of two tagged shoots per plant from the leaf axil to the apical bud. Fresh and dry weights were recorded at the end of each replication (week 6). Leaves, stems (including axillary branches and shoots), and roots were weighed. Roots were separated from the rooting medium by carefully washing them in tap water. Tissue samples were oven-dried to a constant weight at 70°C and weighed.

Statistical Analysis

Treatments were arranged as a 3 (species) x 5 (soil temperature) factorial and were replicated and blocked over time in a randomized complete block design (RCBD) with four replications. Data were analyzed using analysis of variance, multiple comparison tests, repeated measures, and covariance procedures using SAS (SAS Institute, Cary,

N.C.) statistical software. Regression analysis was also done using SigmaPlot program (SPSS Science, Chicago, Ill).

Results and Discussion

Physiological and growth responses to soil temperatures differed among *Annona* species. Almost all variables evaluated were significantly affected by soil temperature ($P \leq 0.05$). Therefore, to compare species effects, physiological and growth variables are reported separately at each soil temperature. The relationship between soil temperature and some physiological and growth variables are shown only for the last measurement date (week 6).

Physiological Measurements

Within 3 weeks after treatments were initiated, leaf chlorophyll index (SPAD readings) of soursop decreased as soil temperatures decreased, and that pattern was consistent throughout the 6 week experiment, when leaf chlorophyll index was lowest at 5 to 10°C, and highest at 35°C (Fig. 3-1). Pond apple and ‘Gefner’ atemoya on sugar apple rootstock showed similar leaf chlorophyll index patterns to soursop over time (data not shown).

Similarly, the leaf chlorophyll index increased as soil temperature increased for all species by week 6, with a stronger relationship for pond apple and soursop ($r^2 = 0.99$), than for ‘Gefner’ atemoya on sugar apple rootstock ($r^2 = 0.81$) (Fig. 3-2). The lowest leaf chlorophyll index was found at 5 and 10°C for all species. Thus, low soil temperatures (5 to 10°C) presumably reduced leaf chlorophyll concentration because leaf chlorophyll concentration of annona leaves is linearly related to leaf SPAD readings (Schaper and Chacko, 1991).

Subtropical and tropical crops, including annonas, are susceptible to chilling injury (Khairi and Hall, 1976; George and Nissen, 1992; Whiley et al., 1999). Exposure to air or soil temperatures below 10°C may cause irreversible chilling injury leading a breakdown of cellular components. The loss of thylakoid membrane fluidity of the leaf chloroplast in many plant species by chilling has been reported (Bowers, 1994). The chloroplast membranes of all higher plants contain trienoic fatty acids, which ensure the maintenance of chloroplast structure when plants are exposed to low temperatures (<10°C). Chilling sensitive plants have a low proportion of these fatty acids (Routaboul et al., 2000). Although cytokinin concentration was not measured in this study, it may be related to the low leaf chlorophyll index observed at soil temperatures of 5 and 10°C. Suboptimal soil temperatures reduce cytokinin activity (Tromp and Ovaa, 1994) and cytokinins are involved in promoting chloroplast development (Chory et al., 1994).

The high leaf chlorophyll index in soursop induced by the highest soil temperature (35°C) (Fig. 3-1) suggests a stabilization the permeability properties of its thylakoid membranes at high temperature (Havaux et al., 1996). This may be associated with the tropical origin of soursop (Morton, 1987). Many tropical species are evolutionarily adapted to high temperature habitats.

For all species, *A* declined to nearly zero within 1 week of exposure to soil temperatures of 5 or 10°C. During the remaining 5 weeks of the study, *A* remained consistently negative indicating a net carbon loss at those soil temperatures. By week 1, there was a linear correlation between *A* and soil temperatures for all species with r^2 of 0.78, 0.83, and 0.84 for ‘Gefner’ atemoya on sugar apple rootstock, pond apple, and

soursop, respectively (Fig. 3-3). The highest A was observed at soil temperatures of 25 and 35°C.

Three weeks after treatments were initiated, ‘Gefner’ atemoya on sugar apple rootstock and soursoop consistently produced positive linear correlations between A and soil temperature exhibiting the highest values at soil temperatures of 25 and 35°C (Fig. 3-4). However, soursoop had higher A than ‘Gefner’ atemoya on sugar apple rootstock at those soil temperatures. In contrast, pond apple produced a positive quadratic relationship between A and soil temperature with the highest values at a soil temperature of 20°C by week 3.

The results of this study are consistent with the evolutionary centers of origin of the *Annona* species tested. Those of tropical origin such as soursoop and sugar apple rootstock had the greatest A at high soil temperatures (25 to 35°C). Pond apple, the only species tested indigenous to the subtropics, exhibited the greatest A at a 20°C soil temperature. Although atemoya is a hybrid between a subtropical (*A. cherimola* Mill.) and a tropical (*A. squamosa* L.) species (Morton, 1987), its response to soil temperatures was more similar to the responses of the tropical species, as evidenced by the highest A and leaf chlorophyll index at 25 and 35°C. These responses suggest that atemoya was influenced by its sugar apple rootstock, which has a tropical origin (Morton, 1987).

The lowest A for all species was observed at the same low soil temperatures (5 to 10°C) at which the lowest leaf chlorophyll index was also found (Fig. 3-2). This suggests that the carboxylation efficiency of leaves was inhibited as a result of reduced leaf chlorophyll content at low soil temperatures. Additionally, the negative A observed at soil temperatures of 5 and 10°C may be due to chilling (< 10°C), which also inhibits directly

A (Taylor and Rowley, 1971; Whiley et al., 1999). If the chilling occurs in the presence of light, there can be photoinhibitory damage to photosystem 2 (PSII) (Bowers, 1994). Photoinhibitory damage can be caused by air chilling temperatures (Powless, 1984; Barth and Krause, 1999) or root chilling temperatures (Delucia et al., 1991). The reduced photosynthetic activity of *Annona* species tested in this study at low soil temperatures may also have been caused, in part, by alteration in the synthesis of ribulose biphosphate carboxylase (Rubisco) since the expression of genes that code for Rubisco may be inhibited as consequence in changes in root sink strength for photoassimilates (Drake et al., 1997; Koch, 1996). Root sink strength is reduced as consequence a slow root growth caused by suboptimal root temperature (Delucia, 1986).

For pond apple the tendency of *A* to decrease after it reached maximum values at 20°C may be attributed to its subtropical origin because photosynthesis is the most sensitive physiological process to high temperature for non-tropical species. Subtropical species exposed to supraoptimal temperatures may exhibit a general breakdown of cellular components such as the loss of semi-permeability of the cell membranes (Björkman et al., 1980).

Net CO₂ assimilation of the *Annona* species tested at soil temperature of 25°C were comparable to the values found by Núñez-Elisea et al. (1999), but lower than those values found for atemoya under orchards conditions by Marler et al. (1994). In general, *A* of many tropical fruit crops is lower for container-grown plants than for trees in orchards. This response has been attributed to a feedback inhibition of *A* as a result of root restriction in the containers (Schaffer et al., 1999).

It appears that A responses of *Annona* species are similar to either soil or air temperatures since the A responses of atemoya on sugar apple rootstock in this study were similar to those found for sugar apple seedlings by Higuchi et al. (1998b), where A was lower at air temperatures of 20/15°C (day/night) than at 30/25°C (day/night) temperatures. Those researchers suggested that high A of sugar apple at high temperatures (30/25°C day/night) was due to the relatively constant temperature of the leaves.

In this study, high A of 'Gefner' atemoya on sugar apple rootstock at 25 and 35°C may imply a physiological acclimation of its photosynthetic apparatus. This may be associated with the high leaf chlorophyll index observed at soil temperatures of 25 and 35°C (Fig. 3-2) since g_s of 'Gefner' atemoya on sugar apple rootstock did not show significant responses to soil temperatures (data not shown). George and Nissen (1992) reported that atemoya exhibits osmotic adjustment. Osmotic adjustment helps maintain both turgor and cell volume of stomata. Thus, g_s and A can be fully to partially maintained (Turner et al., 1978).

Although transpiration (E) of soursop was linearly correlated with soil temperatures from 5 to 35°C 3 weeks after treatments were initiated, g_s did not show significant responses to soil temperatures (data not shown). The response of g_s in *Annona* species has been directly related to RH. Values of RH < 60% reduce g_s (George et al., 1990), and the RH in this study ranged 80 to 90%.

For the last replication of the study (October-November), there was an unseasonably cold period for nearly a week, which decreased A values for all treatments (data not shown).

Growth Measurements

Growth rate of the *Annona* species tested varied over time depending on soil temperatures.

Plant height of soursop increased linearly ($r^2 = 0.91$) as soil temperatures increased from 5 to 35°C (Fig. 3-5). Plants grown at soil temperatures of 5 and 10°C exhibited less growth than plants grown at the other soil temperatures. The height of ‘Gefner’ atemoya on sugar apple rootstock was also linearly correlated with soil temperatures ($r^2 = 0.61$). Pond apple height was quadratically correlated with soil temperatures ($r^2 = 0.44$), with the greatest height occurring at soil temperatures of 25°C (Fig. 3-5). However, differences in height were small among soil temperatures.

The greatest shoot growth of soursop occurred from June to August. Soursop shoot length was lowest at low soil temperatures (5 to 10°C) and high soil temperature (35°C) promoted the greatest shoot growth (data not shown). Shoot growth of pond apple and ‘Gefner’ atemoya on sugar apple rootstock were less affected by soil temperatures than that of soursop (data not shown).

There was a strong quadratic relationship ($r^2 = 0.99$) between soil temperatures and trunk diameter of soursop measured 5 weeks after the treatments were initiated (Fig. 3-6). Soursop trees at soil temperatures 5 or 10°C had smaller trunk diameters than trees at the other soil temperatures. The relationships between soil temperature and trunk diameter were linear for ‘Gefner’ atemoya on sugar apple rootstock and quadratic for pond apple, with the greatest trunk diameter at soil temperature of 35°C for both species (Fig. 3-6).

After 6 weeks in the root chambers, the number of leaves per shoot varied among *Annona* species (Fig. 3-7). In general, soil temperatures had no effect on the number of leaves per shoot of pond apple. ‘Gefner’ atemoya on sugar apple rootstock tended to have

the lowest leaf number per shoot at the 5°C soil temperature but it did not show significant differences between number of leaves and the other soil temperatures. Soursop tended to have the greatest leaf number per shoot at 35°C.

The relationship between soil temperatures and leaf dry weight was linear within the temperature ranges tested for 'Gefner' atemoya on sugar apple rootstock and quadratic for pond apple (Fig. 3-8A). 'Gefner' atemoya on sugar apple rootstock had the lowest leaf dry weight at 5°C and the highest at 35°C. In contrast, the maximum leaf dry weight occurred at the 20°C soil temperature for pond apple. There was no significant effect of soil temperature on leaf dry weight of soursop (data not shown). The relationship between soil temperatures and stem dry weight was linear for 'Gefner' atemoya on sugar apple rootstock and soursop (Fig. 3-8B). There was no significant effect of soil temperatures on stem dry weight of pond apple (data not shown). Root dry weight increased linearly with soil temperatures for 'Gefner' atemoya on sugar apple rootstock and soursop, with the lowest values at 5°C and the highest values at 35°C for both species (Fig. 3-8C). For pond apple, there was a strong quadratic relationship ($r^2 = 0.98$) between soil temperature and root dry weight, with the lowest values at 5°C and the highest values at 20 and 25°C. In general, leaf, stem, and root fresh weights of all species showed similar patterns to the dry weights (data not shown).

Sub or supraoptimal soil temperatures can cause changes in some physiological process in the root, such as respiration and carbohydrate metabolism (Gur et al., 1972; Delucia, 1986; Du and Tachibana, 1994), and synthesis of certain phytohormones such as cytokinins (Itai et al., 1973; Tromp and Ovaa, 1994; Tachibana et al., 1997; Dieleman, et al., 1998), which can result in decreased plant growth.

Plants grown at 5 and 10°C soil temperatures exhibited negative A within the first week of the study, indicating that plants were photorespiring, and were not fixing CO_2 . Consequently plant growth was significantly decreased at those temperatures. Suboptimal or supraoptimal root temperatures not only reduce root dry-matter accumulation of *Annona* species but also the whole plant dry-matter accumulation (George and Nissen, 1987). This response may be due to a feedback inhibition from the source (leaves) as a result of reduced sink strength (slow root growth) (Delucia, 1986). Reduced growth caused by low temperature is also associated with limited water uptake. The viscosity of water decreases at low temperatures, which has been reported in many crops (Markhart et al., 1979; Syvertsen et al., 1983; Wilcox et al., 1983). Reduced water uptake leads to reduced ion uptake and reduced plant growth. Additionally, a reduced cytokinin activity caused either by suboptimal (Tromp and Ovaa, 1994) or by supraoptimal root temperatures (Itai et al., 1973) reduce plant growth since cytokinins promote meristematic growth (cell division).

George and Nissen (1987) reported higher dry matter accumulation at root temperatures of 15°C than at 28°C for most cherimoya cultivars. In contrast, they found a significant increase in dry matter accumulation of pond apple and sugar apple trees grown at root temperatures of 28°C compared to those exposed to root temperatures of 15°C. Thus, the impact of suboptimal or supraoptimal soil temperatures on *annona* growth depends on the species.

Conclusions

Soursop and sugar apple, which originated in tropical climates, were less tolerant to low soil temperatures than pond apple, which is indigenous to subtropical areas. Thus, the only two known flood-tolerant *Annona* species, soursop and pond apple (Núñez-

Elisea et al., 1998, 1999) require different soil temperatures for optimum growth. Soursop was best adapted to 25 to 35°C and pond apple to 20 to 25°C. This information may help in selecting or developing flood-tolerant annona rootstocks adaptable to a wide range of soil temperatures.

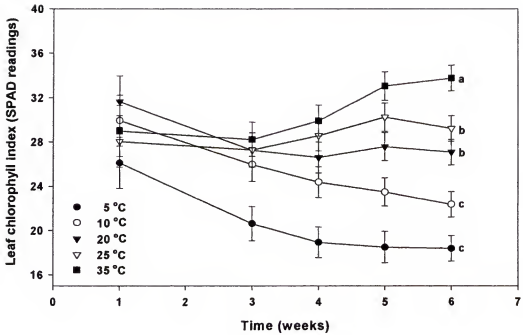


Figure 3-1. Effect of soil temperatures on leaf chlorophyll index of soursop over a 6-week period. Symbols represent means \pm SE of 4 replications, with 2 plants per replication and 6 leaf samples per plant. Different letters indicate significant differences among means by LSMEANS ($P \leq 0.01$) on the last measurement date (week 6).

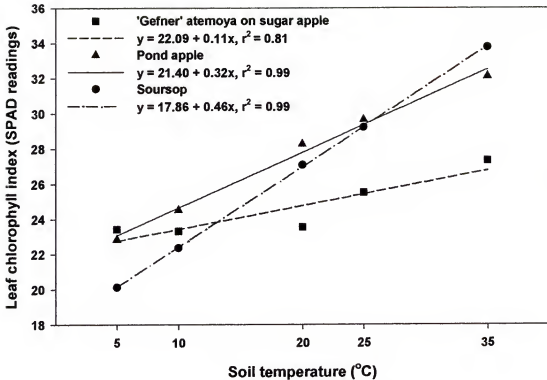


Figure 3-2. Soil temperatures and leaf chlorophyll index of *Annona* species 6 weeks after temperature treatments were initiated. Symbols represent the means of 4 replications, with 2 plants per replication and 6 leaf samples per plant.

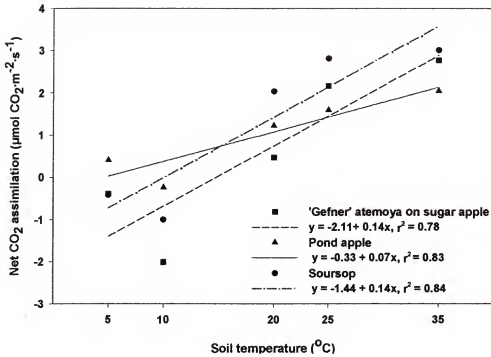


Figure 3-3. Soil temperatures and net CO₂ assimilation of *Annona* species 1 week after temperature treatments were initiated. Symbols represent the means of 3 replications with 2 plants per replication.

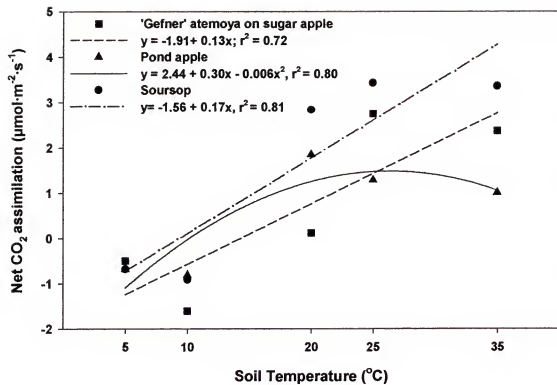


Figure 3-4. Soil temperatures and net CO₂ assimilation of *Annona* species 3 weeks after temperature treatments were initiated. Symbols represent the means of 3 replications with 2 plants per replication.

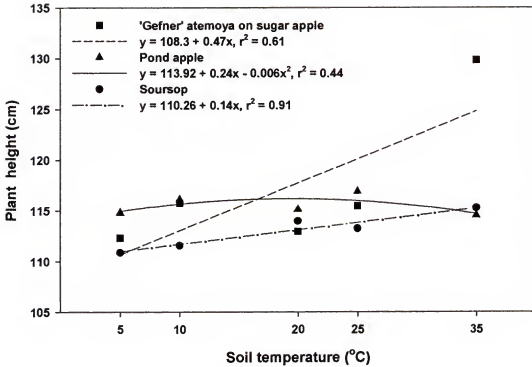


Figure 3-5. Soil temperatures and plant height of *Annona* species 6 weeks after temperature treatments were initiated. Symbols represent the means of 4 replications with 2 plants per replication.

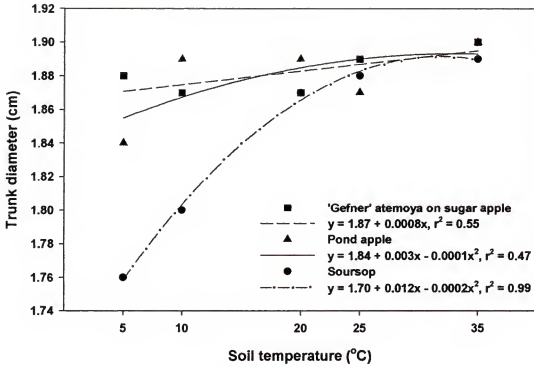


Figure 3-6. Soil temperatures and trunk diameter of *Annona* species 5 weeks after temperature treatments were initiated. Symbols represent the means of 4 replications with 2 plants per replication.

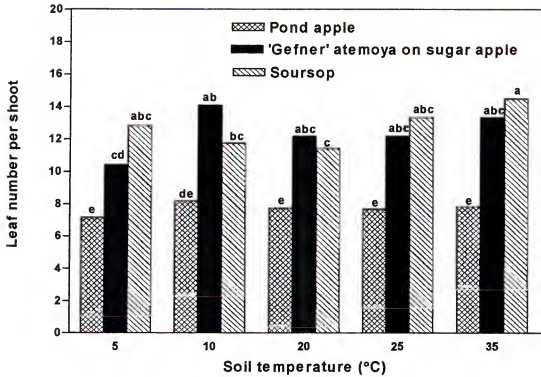


Figure 3-7. Soil temperatures and leaf number per shoot of *Annona* species 6 weeks after temperature treatments were initiated. Symbols represent the means of 4 replications with 2 plants per replication and 2 shoot samples per plant. Different letters within soil temperatures indicate significant differences among species by LSMEANS, $P \leq 0.01$.

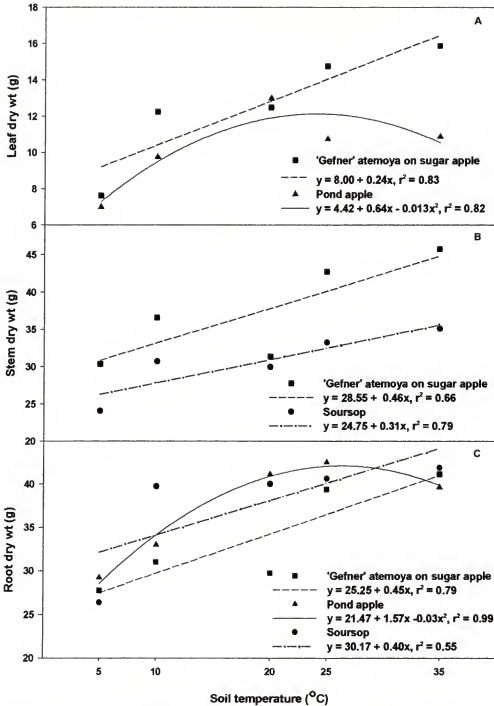


Figure 3-8. Soil temperatures and leaf (A), stem (B), and root dry weights (C) of *Annona* species 6 weeks after temperature treatments were initiated. Symbols represent the means of 4 replications with 2 plants per replication. Stem dry weight of pond apple and leaf dry weight of soursop were not significantly different ($P < 0.05$).

CHAPTER 4
FLOODING, SOIL TEMPERATURE, PHYSIOLOGY, AND GROWTH OF TWO
Annona SPECIES IN CONTAINERS

Introduction

The genus *Annona* includes several subtropical and tropical fruit tree species with economic importance throughout the world (Morton, 1987; Nakasone and Paull, 1998). The commercially important species include cherimoya (*A. cherimola* Mill.), soursop (*A. muricata* L.), sugar apple (*A. squamosa* L.), and atemoya (*A. squamosa* L. x *A. cherimola* Mill). Pond apple (*A. glabra* L.) is a species of *Annona* native to tropical and subtropical wetlands of the Americas, and is generally not considered a commercial crop (Nakasone and Paull, 1998).

Commercial fruit crops in the *Annonaceae* such as sugar apple and atemoya are susceptible to flooding damage (Núñez-Elisea et al., 1998, 1999). In contrast, soursop and pond apple are relatively tolerant to flooding. Thus, soursop and pond apple have the potential to be used as flood-tolerant annona rootstocks (Núñez-Elisea et al.; 1998, 1999). The most notable effect of flooding on crops is a reduction in root and shoot growth due to decreased soil oxygen content (Schaffer et al., 1992). Various physiological and metabolic processes are also negatively affected by flooding, including decreases in net CO₂ assimilation (*A*) (Núñez-Elisea et al., 1999; Zude-Sasse et al., 1998; Larson et al., 1991a), stomatal conductance to CO₂ (*g_s'*) (Davies and Flore, 1986; Crane and Davies, 1987), transpiration (*E*) (Davies and Flore, 1986; Crane and Davies, 1989), and root hydraulic conductivity (Syvertsen et al., 1983; Crane and Davies, 1987). Flooding of

commercial *Annona* species, even for short periods, reduces growth, may cause defoliation, and severely reduces flowering and fruit set (Marler et al., 1994).

Some areas where annonas are grown commercially such as south Florida are periodically subjected to low winter temperatures ($<10^{\circ}\text{C}$). The effect of low air temperature is more pronounced for soursop than for atemoya, sugar apple, or pond apple since branches of juvenile soursop plants are killed when air temperatures are below 0°C (Campbell et al., 1977). There is a general lack of published information about the effects of suboptimal temperature on root function of soursop and pond apple. Therefore, the impact of the soil temperature and flooding on growth and development of these species should be determined before recommending them as rootstocks for flood-prone subtropical areas.

The objective of this study was to evaluate the effect of soil temperatures on physiology and growth of flooded and non-flooded pond apple and soursop.

Materials and Methods

The study was conducted from August 2001 to June 2002 in a sunlit glasshouse at the University of Florida in Gainesville, Fla. Average day/night air temperatures during the experimental period ranged from $40/25$ to $25/15^{\circ}\text{C}$ and relative humidity averaged 80 to 95%.

Plant Material

Seedling trees between 6 months and 1.5 years of age were used in this study. Seedling trees of pond apple (*A. glabra* L.) and soursop (*A. muricata* L.) were grown in 3.8-L containers in well-drained media (40% Florida peat moss, 20% vermiculite, and 40% pine bark by volume). Plants were irrigated daily to container capacity, and

fertilized monthly with 3.7 g L^{-1} of water-soluble fertilizer (14.5N-8P-16.6K; with ammonium, nitrate, and urea as N sources).

Treatments

Six weeks prior to initiating the treatments all plants were pruned to produce new growth flushes. Two uniform trees of each species were randomly placed in each of five root temperature chambers, and subjected to one of five soil temperatures (5, 10, 20, 25, or $35 \pm 2^\circ\text{C}$). There was one root temperature chamber for each soil temperature treatment and the chambers were used for the same temperatures for each replication. Plants at each temperature were either non-flooded (control) or continuously flooded. There was a single-plant sample of each species in each treatment combination (flooding/soil temperature). Due to the limited number of root temperature chambers, treatments were replicated and blocked over time for a total of 4 replications: August-September, October-November, April-May, and May-June. Twenty plants were used in the study for each replication (block). Plants of each replication were subjected to the treatments for 6 weeks based on previous study (See Chapter 3 of this dissertation). In that previous experiment the effects of soil temperatures on physiology and growth of pond apple and soursop were significantly different by week 6

Root Chambers

The root temperature chambers used for the experiment were thermostatically controlled freezers, which were modified by removing the lids and replacing them with styrofoam lids. The styrofoam lids allowed for potted plants to be positioned with roots in the controlled-temperature chamber and canopy exposed to ambient air temperature. The top of each plant container was insulated with styrofoam similar to that used for the lids of the root temperature chambers to reduce evaporation from the potting media and

moderate temperature changes, while allowing for adequate root aeration. This approach was previously used for carambola (*Averrhoa carambola* L.) (George et al., 2002).

A two-speed oscillating fan was placed inside each of the growth chambers to help maintain uniform temperatures throughout the chamber and to allow for air circulation. In the 5, 10, and 20°C root temperature chambers, five 20-L plastic buckets filled with water were placed inside to help maintain constant temperatures. For the chambers at 25 and 35°C, the entire chambers were half-filled with water to help maintain constant temperature. A VISI-THERM aquarium water heater (Aquarium Systems, Mentor, OH) was installed below the water level inside the 35°C chamber to maintain the temperature. Ambient air temperature in the glasshouse and soil temperatures in each root chamber were continuously monitored and recorded with a Hobo H8 Pro Series temperature logger (Onset Computer Corporation, Pocasset, Mass.) and a Fisher mercury thermometer (Fisher Scientific Co. LLC, Ill.). The thermometers monitoring soil temperatures were positioned at the center of the rooting medium in one of the containers in each root chamber. Prior to their installation, the temperature loggers were calibrated by comparing temperatures to temperatures obtained with a mercury thermometer in ice water and in water at ambient temperature. Ambient air temperatures and RH in the glasshouse were measured at a height 1.82 m above ground level.

Soil Measurements

Soil redox potential (Eh) was monitored with a platinum combination electrode (Ag^+/AgCl , Accumet, Fisher Scientific, Pittsburgh, Pa.) attached to a portable pH meter (Accumet AP62, Fisher Scientific, Pittsburgh, Pa.). Soil Eh was recorded for each flooded plant for the first 3 d of each replication over time (block), and then 7 d after flooding.

Physiological Measurements

Electrolyte leakage was measured from 0.5 g of fibrous roots (fresh weight) at the end of each replication over time (block). The roots were cut from each plant, rinsed in deionized water, and placed in test tubes containing 15 ml of deionized water. Test tubes were shaken for 1 h at room temperature (25°C), and conductivity measurements of the effusate were determined using a conductivity bridge (YSI, 31A, YSI Incorporated Yellow Springs, OH) and an electrode (YSI, 3403, YSI Incorporated Yellow Springs, OH). Roots were then frozen at -20°C for a minimum of 12 h, boiled for 30 min, cooled to room temperature, and conductivity was again measured (Crane and Davies, 1987).

Leaf chlorophyll index was determined with a chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd, Japan). Leaf chlorophyll measurements were made on six randomly selected leaves per plant. Leaf chlorophyll index was measured on the same leaves used for gas exchange measurements.

Net CO₂ assimilation (A), stomatal conductance for CO₂ (g_s'), and transpiration (E) were measured weekly using a portable infrared gas analyzer (LCA-2, Analytical Development Co. LTD, England). Measurements were made between 1200 and 1500 HR at a photosynthetic photon flux (PPF) $>700 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which is above light saturation for annona photosynthesis (Marler and Zozor, 1996; Utsunomiya and Higuchi, 1996) with sunlight as the light source. The leaves selected for gas exchange measurements were the most recently matured, fully exposed sunlit leaves located at the 4th or 5th node below the shoot apex.

Growth Measurements

Plant height, trunk diameter, total number of leaves, leaf abscission, and fresh and dry weights were determined for all plants. All growth measurements except trunk

diameter and, fresh and dry weights were made at the beginning (day 1) of each replication and repeated weekly. Trunk diameter was recorded every 2 weeks. Plant height was determined on the main stem and was measured from the surface of the planting medium to the midpoint of the apical bud. Trunk diameter was measured at a 10-cm height above the surface of the planting medium using a micrometer caliper. Leaf abscission was expressed as percentage of the total number of leaves yellowing and abscising from the 10 lowest leaves from the base of the main stem.

Fresh and dry weights were determined at the end of each replication. Leaves, stems (including axillary branches and shoots), and roots were weighed. Roots were separated from the rooting medium by carefully washing them in tap water. Tissue samples were oven-dried to a constant weight at 70°C and weighed.

Statistical Analysis

Treatments were arranged as a 2 (species) x 5 (soil temperature) x 2 (flooding treatments) factorial design, with soil temperature and flooding treatments as main effects. Treatments were replicated and blocked over time in a randomized complete block design (RCBD) with four replications. Data were analyzed by a combination of statistical tests, including analysis of variance, multiple comparison, repeated measures, and covariance procedures using SAS (SAS Institute, Cary, N.C.) statistical software. Regression analysis was also done using SigmaPlot Program (SPSS Science, Chicago, Ill.).

Results and Discussion

Soil Redox Potential (Eh)

There were no differences in soil Eh between the species (data not shown); therefore all data from both species were pooled. For all soil temperatures, soil Eh was

approximately 360 mV on day 1 when treatments were initiated (Fig. 4-1). Thereafter, an inverse relationship existed between soil temperatures and soil Eh for the flooded treatments. After 2 d of flooding, soil Eh in each of the 35, 25, 20, and 10°C soil temperatures was lower than 200 mV, indicating anaerobic soil conditions (Ponnamperuma, 1984). Soil Eh at 5°C decreased slower than at the other soil temperatures. These results are similar to those of Larson et al. (1991b) who observed a more rapid soil reduction at soil temperatures of 22.5 and 30°C than at 15°C in mango. This response was most likely due to increases in root respiration and microbial activity and rapid O₂ depletion at high soil temperatures. After 7 d of flooding, soil Eh in all soil temperature treatments was less than 100 mV indicating very low O₂ availability to the roots. Soil Eh was measured again at 7 d after flooding because it sometimes tends to increase before obtaining a stable potential (Larson et al., 1991c).

Morphological Adaptations to Flooding

Some plants can adapt to flooding stress either by avoiding oxygen deficits by developing aerenchyma, adventitious roots or hypertrophied (swollen) trunk lenticels for improved root gas exchange, or by physiologically adapting to oxygen deficits (Schaffer et al., 1992; Armstrong et al., 1994; Crawford and Braendle, 1996). In this study, pond apple developed hypertrophied (swollen) trunk lenticels at 20, 25, and 35°C soil temperatures within 1 week of flooding with the most lenticels developing at 25°C (data not shown). Often, flooded pond apple plants exposed to 5 and 10°C soil temperatures exhibited leaf epinasty within 2 weeks after flooding treatments were initiated. These epinasty symptoms occurred earlier at 10 than at 5°C. Additionally, pond apple developed adventitious roots, upward root growth through the soil from preexisting roots, and basal trunk swelling in responses to flooding. Soursop plants also developed some

hypertrophied trunk lenticels at 20 and 25°C, however, lenticel development was most pronounced at 35°C (data not shown). Fewer lenticels were observed for soursop than for pond apple. Soursop plants produced some vegetative shoots at the base of the trunk and some plants developed adventitious roots at 20 and 25°C. Only the plants of both species with visible morphological adaptations survived extended flooding. Thus, within 4 weeks of flooding, 100 % of pond apple and soursop plants at 5 and 10°C soil temperatures showed symptoms of flooding damage such as leaf wilting and necrosis followed by defoliation, and finally death. Development of morphological adaptations to flooding in pond apple and soursop appears to be a temperature-dependent process since no morphological adaptations were observed in plants at 5 or 10°C soil temperatures. These morphological changes in response to flooding, also reported previously in pond apple and soursop (Núñez-Elisea et al., 1998), were presumably adaptations to flooding since there was 100% plant survival at 20, 25, and 35°C soil temperatures after 6 weeks of flooding. Hypertrophied trunk lenticels may allow internal oxygen diffusion to flooded roots (Armstrong, 1968; Kozłowski, 1984; Jackson and Attwood, 1996) and/or function as excretory sites for potentially toxic metabolites formed in the roots during anaerobic respiration (Chirkova and Gutman, 1972). Similarly, basal trunk swelling is thought to increase gas diffusion because there is an increased porosity among stem tissues, thus enhancing internal aeration (Armstrong et al., 1994; Yamamoto et al., 1995). Adventitious roots may help to maintain continuous nutrient and water absorption (Sena and Kozłowski, 1980). The production of new vegetative shoots at the base of the trunk of soursop may contribute to O₂ absorption since the normal root supply-route of O₂ absorption was limited by flooding (Vartapetian and Jackson, 1997).

There were significant interactions ($P \leq 0.05$) between flooding and soil temperatures for all physiological and growth variables measured. Therefore, for each soil temperature treatment effects are reported separately for each flooding treatment. The relationship between soil temperature and physiology or growth is shown only at the last measurement date (week 4 or 6).

Physiological Measurements

Root electrolyte leakage

There were linear and quadratic relationships between root electrolyte leakage and soil temperatures for non-flooded pond apple and soursop plants, respectively (Fig. 4-2A). The highest root electrolyte leakage was found in plants of each species exposed to 5 and 10°C soil temperatures, but was higher for soursop than for pond apple. For each species, flooded plants had more root electrolyte leakage than non-flooded plants (Fig. 4-2A and B), and soursop had more root electrolyte leakage than pond apple. The least electrolyte leakage occurred in flooded soursop plants at soil temperatures of 20 and 25°C. These results are consistent with the putative subtropical and tropical origins of pond apple and soursop (Morton, 1987; Nakasone and Paull, 1998), respectively, since the highest values of root electrolyte leakage were found at relatively low soil temperatures of 5 and 10°C. Subtropical and tropical species are susceptible to chilling damage ($< 10^\circ\text{C}$), which causes a loss of semi-permeability of cell membranes (Björkman et al., 1980). In addition, flooded pond apple, which is native to wetland areas, had lower root electrolyte leakage values than soursop over a range of flooded soil temperatures further attesting to its superior flood tolerance over soursop.

Flooding also affects cellular processes, changes permeability of root cell membranes, and consequently reduces the supply of available energy. The ultimate stage

of cell survival under anoxia is reached when sub-cellular compartmentalization is disrupted (Rawyler et al., 1999). Progressive membrane damage has been related to an extensive hydrolysis of the root membrane lipids in flood-sensitive species under anoxia (Kolb et al., 2002). Membrane disruption involves not only losses of cations, such as K^+ and basic metabolites, but also the concurrent loss of balancing anions, such as Cl^- , organic acids and other acidic metabolites (Buwalda et al., 1988). The increased root electrolyte leakage observed in flooded soursop at 5 and 10°C compared with non-flooded plants at the same temperatures suggests that flooding caused the membrane disruption of soursop root cells leading to loss of cations and anions. Crane and Davies (1987) observed that electrolyte leakage from roots of rabbiteye blueberry occurred 6 d after flooding followed by decreases in stomatal and hydraulic conductances. The results with rabbiteye blueberry suggested that the loss of anions and cations (K^+) altered the electrostatic balance and cell turgor, and consequently stomata conductance decreased since stomatal opening is regulated by K^+ balance.

Leaf chlorophyll index

As soil temperatures increased, the leaf chlorophyll index generally increased for non-flooded plants of both species from 5 to 35°C by week 4 (Fig. 4-3A). The lowest leaf chlorophyll index was at soil temperatures of 5 and 10°C. A highly significant linear relationship exists between extractable leaf chlorophyll concentration and leaf SPAD readings from annona leaves (Schaper and Chacko, 1991). Thus, low soil temperatures reduced leaf chlorophyll concentration.

For flooded plants, there were quadratic and linear relationships between soil temperatures and the leaf chlorophyll index for pond apple and soursop, respectively, by week 4 (Fig. 4-3B). For flooded pond apple, the leaf chlorophyll index increased as soil

temperatures increased from 5 to 25°C, with a decrease at 35°C. In contrast, flooding reduced the leaf chlorophyll index of soursop at all soil temperatures compared with non-flooded treatment. Reduced leaf chlorophyll index for non-flooded and flooded plants of both species at soil temperatures of 5 and 10°C may be due to the high root electrolyte leakage observed at those temperatures (Fig. 4-2A and B). Root electrolyte leakage involves the loss of cations and anions such as K^+ and Cl^- (Buwalda et al., 1988) and may cause a leaf nutritional imbalance and leaf chlorosis.

Similarly, the reduced leaf chlorophyll content of soursop as a result of flooding may have been partially due to a higher root electrolyte leakage in flooded than non-flooded soursop plants. Magnesium is one of the main cations leaked during stress (Buwalda et al., 1988), and constitutes one of the main components of the chlorophyll molecule. In contrast, flooding did not decrease leaf chlorophyll index of pond apple plants. The differential responses between soursop and pond apple may be associated in part with the wetland origin of pond apple (Morton, 1987; Nakasone and Paull, 1998), since morphological adaptations to flooding such as adventitious root and hypertrophied trunk lenticel development occurred more frequently in pond apple than soursop. For instance, more adventitious roots may partially increase nutrient and water absorption (Sena Gomes and Kozlowski, 1980), thus preventing leaf chlorosis in pond apple. Similarly, the development of hypertrophied trunk lenticels may facilitate, in part, O_2 diffusion to flooded roots of pond apple (Kozlowski, 1984) and produce energy to maintain ion uptake. Zotz et al. (1997) reported that pond apple has an efficient hydraulic system, which may help maintain nutrient uptake and transport under flooded conditions (Huang, 2000), since nutrient translocation is dependent on the transpiration stream. In

field trials in calcareous soils, pond apple plants under non-flooded conditions exhibited Fe deficiency (B. Schaffer, personal communication) suggesting that pond apple may have a high Fe requirement. Short-term flooding can increase available Fe in the soil (Larson et al., 1991c; Zude-Sasse et al., 1998; Zude-Sasse and Schaffer, 2000), which may turn in increase chlorophyll content since Fe is involved in chlorophyll synthesis and maintains chlorophyll structure and function (Abadía, 1992).

Leaf gas exchange

Non-flooded pond apple and soursop plants exposed to soil temperatures of 5 or 10°C had negative A , indicating a net respiratory loss of carbon within 1 week of initiating treatments (Fig. 4-4A and B) that remained for the next 2 weeks. By week 6, A of non-flooded plants of both species tended to increase as soil temperatures increased, although there were no significant differences in A among soil temperatures. Flooded pond apple plants exposed to soil temperatures of 5 or 10°C also exhibited negative A values within 1 week after flooding (Fig. 4-4C), although A was lower in flooded than in non-flooded plants by week 3. For pond apple plants at soil temperatures of 20, 25 or 35°C, there was no effect of flooding on A (Fig. 4-4A and C). Núñez-Elisea et al. (1999) also found that pond apple plants subjected to cyclic or continuous flooding for 50 d had A comparable to those of non-flooded controls. In this study, comparable A values in non-flooded and flooded plants of pond apple are consistent with the leaf chlorophyll index observed in those plants since flooding did not reduce leaf chlorophyll content of pond apple (Fig. 4-3A and B). These results suggest that A of pond apple was mainly regulated by non-stomatal limitations since in general there were no significant differences in g_s and E among soil temperature or flooding treatments (data not shown). Zotz et al. (1997) observed low whole-plant resistance to water flow in pond apple in its

natural environment, which may increase water movement from soil to leaves assuring availability of water for photosynthesis. Thus, water stress may not limit photosynthesis in flooded pond apple trees.

Although all flooded soursop plants survived at soil temperatures of 20, 25 or 35°C (data not shown), *A* of flooded plants was considerably lower than for non-flooded plants (Fig. 4-4B and D). Reduced *A* of flooded soursop plants may be associated with high root electrolyte leakage (Fig. 4-2B) and reduced leaf chlorophyll content (Fig. 4-3B) induced by flooding. In general there were no significant differences in g_s and *E* caused by soil temperature and flooding (data not shown), suggesting that *A* of soursop was mainly reduced by non-stomatal limitations.

Growth Measurements

Leaf abscission

For each species, there was a negative relationship between soil temperatures and leaf abscission in each flooding treatment. For both species, the most leaf abscission was observed at soil temperatures of 5°C for non-flooded plants and at 5 and 10°C for flooded plants, although flooded plants had higher leaf abscission (~ 60%) than non-flooded plants (~ 40%) (data not shown). Leaf abscission in soursop seems to be a very sensitive response to either low soil or air temperatures. Popenoe (1974) also reported that air temperatures between 5 to 7°C induced leaf abscission in soursop.

Epinasty was observed before leaf senescence in flooded pond apple plants at soil temperatures of 5 and 10°C. Thus, the combination of flooding and suboptimal soil temperatures caused leaf abscission. Ethylene appears to be the primary regulator of leaf epinasty (Brandford and Yang, 1980) and abscission (Banga et al., 1997) as a result of flooding (Gur et al., 1998; Grinhko and Glick, 2001). Additionally, flooding and

temperature stresses both reduce the synthesis of cytokinins (Reid and Bradford, 1984; Tromp and Ova, 1994), which are generally known to inhibit leaf senescence (Smart, 1994). Thus, a reduced synthesis of cytokinins may have promoted leaf abscission of pond apple and soursop, especially at floodwater temperatures of 5 and 10°C.

Trunk diameter

For non-flooded plants, the relationships between soil temperature and trunk diameter was linear for pond apple and quadratic for soursop plants (Fig. 4-5A). The largest trunk diameter occurred at the 35°C for pond apple and at 25 and 35°C for soursop. A morphological response of pond apple to flooding was thickening of the trunk base (Fig. 4-5A and B), with the most thickening occurring at 25 and 35°C. In contrast, the trunk diameter of soursop plants did not increase as a result of flooding (Fig. 4-5A and B).

Núñez-Elisea et al. (1998, 1999) obtained similar results for flooded pond apple plants, which exhibited nearly a 60% increase in trunk diameter 50 d after flooding. The increased trunk diameter of flooded pond apple trees is due to an increase in stem xylem thickness and increased fiber cell radius (Núñez-Elisea et al., 1999).

Plant height, total leaf number, and fresh and dry weights

The height of non-flooded pond apple and soursop plants increased linearly as soil temperatures increased from 5 to 35°C 4 weeks after treatment initiation (Fig. 4-6A). Flooding did not reduce plant height in pond apple and the tallest plants were observed at 25 and 35°C. In soursop, plant height was less for flooded plants than non-flooded plants by week 4 (Fig. 4-6B). From week 4 to week 6, there was not much change in plant height for non-flooded and flooded plants of each species exposed to soil temperatures of 20, 25 or 35°C (data not shown).

For non-flooded plants of each species, the total number of leaves increased as soil temperatures increased linearly from 5 to 35°C by week 4 (Fig. 4-7A), although the relationship was stronger for soursop ($r^2 = 0.85$) than for pond apple ($r^2 = 0.55$). Flooded pond apple plants had the most leaves at 35°C at week 4 (Fig. 4-7B). Flooded soursop plants did not show a significant difference in total leaf number among soil temperatures (data not shown). From week 4 to week 6, there was not much change in total leaf number for non-flooded and flooded plants of each species exposed to soil temperatures of 20, 25 or 35°C (data not shown).

Stem dry weight of flooded pond apple increased linearly with soil temperatures from 5 to 35°C (Fig. 4-8A). There was no significant effect of soil temperature on stem dry weight of non-flooded pond apple (data not shown). As soil temperatures increased, root dry weight of non-flooded and flooded pond apple plants increased from 5 to 35°C (Fig. 4-8B), although flooded plants tended to have lower root dry weights than non-flooded plants. There were no significant effects of flooding on root and stem dry weights of soursop (data not shown). Leaf dry weight of non-flooded plants was linearly correlated with soil temperatures of each species (Fig. 4-9A), although the relationship was stronger for soursop ($r^2 = 0.96$) than for pond apple ($r^2 = 0.66$). For flooded pond apple, the highest leaf dry weight was observed at soil temperatures of 25 and 35°C and for soursop at 35°C (Fig. 4-9B). Leaf, stem, and root fresh weights of both species showed similar patterns to dry weights (data not shown).

In general flooding did not inhibit pond apple growth compared to soursop plants. In its native wetland habitat (Morton, 1987; Nakasone and Paull, 1998), pond apple may have evolved the ability to metabolize and grow under restricted O₂ availability, which is

evidenced by the small effect of flooding on leaf chlorophyll index (Fig. 4-3B) and *A* (Fig. 4-4C). Several factors may trigger extension growth in flooded plants. Ethylene may act in concert with other phytohormones such as gibberellins (Raskin and Kende, 1984) or auxins (Rijnders et al., 1996) to promote growth in some flood-tolerant species under flooded conditions. A stimulation of ethylene-induced leaf growth has been observed in *Rumex palustris*, another flood-tolerant species (Voeseinek and Blom, 1989). Núñez-Elisea et al. (1999) also found that flooding did not decrease shoot growth in pond apple but decreased it in soursop.

Flooded pond apple plants had slightly lower root dry weights than non-flooded plants which may have been due to altered partitioning patterns since greater allocation of carbon to above-ground tissue is characteristic of plants subjected to flooding (Tang and Kozłowski, 1982; Megonigal and Day, 1992). In many cases, gases diffuse from shoots to roots when roots have limited O₂ due to flooding (Vartapetian and Jackson, 1997). Low root dry weight as a result of flooding has also been reported in other flood-tolerant species such as *Fraxinus mandshurica* Rupr. (Yamamoto et al., 1995) and *Taxodium distichum* L. (Pezeshki et al., 1996). In contrast to pond apple, flooded soursop plants generally had less plant growth than non-flooded plants, especially at 5 and 10°C soil temperatures. The growth rate of soursop may slow down not only due to decreased availability of water and minerals from oxygen-deficient roots (Drew, 1990; Kozłowski, 1984), but also to invest energy in developing morphological adaptations to flooding for enhanced survival.

Plant growth depends on the balance between C gain during photosynthesis and C loss through respiratory processes. Flooded pond apple and soursop plants grown at soil

temperatures of 5 and 10°C exhibited negative *A* within the first week of the study, indicating a net carbon loss. Consequently plant growth was significantly reduced.

Reduced growth caused by flooding and suboptimal temperatures is also associated with a reduction in water uptake. The viscosity of water decreases at low temperatures, which has been reported in other fruit crops such as citrus (Syvertsen et al., 1983; Wilcox et al., 1983) and blueberry (Crane and Davies, 1989). Reduced water uptake leads to reduced ion uptake. Additionally, flooding reduces soil concentration of P, K (Kozłowski and Pallardy, 1984; Pezeshki et al., 1999), and especially N due to denitrification (Ponnamperuma, 1984), which may have reduced the leaf chlorophyll content in soursop and consequently plant growth.

George and Nissen (1987) found a significant increase in dry matter accumulation in pond apple and sugar apple trees grown at soil temperatures of 28°C compared to those exposed to soil temperatures of 15°C. Thus, the impact of suboptimal soil temperatures on annona growth depends on the *Annona* species since soil temperatures lower than 20°C decreased pond apple and soursop growth.

Conclusions

Pond apple was more flood-tolerant than soursop. Although 100% of both species survived 6 weeks of continuous flooding at soil temperatures of 20, 25 and 35°C, the combination of flooding and soil temperatures of 5 and 10°C caused plant death for both species by week 4. Only plants of both species that exhibited morphological adaptations survived continuous flooding. Optimum growth for non-flooded pond apple was at 25 to 35°C and 20 to 25°C for flooded plants. Soursop exhibited maximum growth at soil temperatures of 35°C for non-flooded conditions and at 25°C for flooded conditions. Soursop may be a flood-tolerant rootstock only for areas subjected to short-term flooding.

The results of this study may help in selecting or developing flood-tolerant annona rootstocks adaptable to a range of soil temperatures between 20 to 35°C since flooding in many agricultural soils is usually transitory.

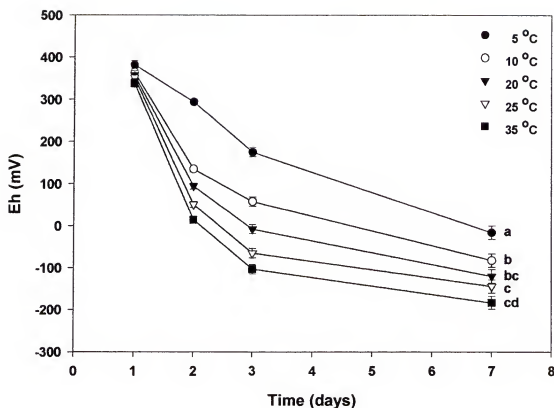


Figure 4-1. Effect of soil temperatures on soil redox potential (Eh) of flooded pond apple and soursop plants (with species pooled) over time. Symbols represent means \pm SE of 4 replications (block), with 1 plant for each species per replication. Different letters indicate significant differences among means by LSMEANS, ($P \leq 0.05$) on the last measurement date (day 7). SE bars not visible are masked by the data symbols.

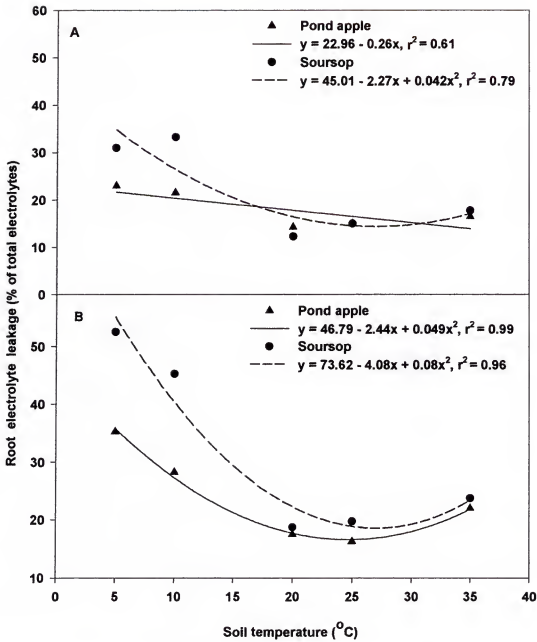


Figure 4-2. Soil temperatures and root electrolyte leakage of (A) non-flooded and (B) flooded *Annona* species 6 weeks after flooding and soil temperature treatments were initiated. Symbols represent the means of 4 replications (block) with 4 root samples per plant.

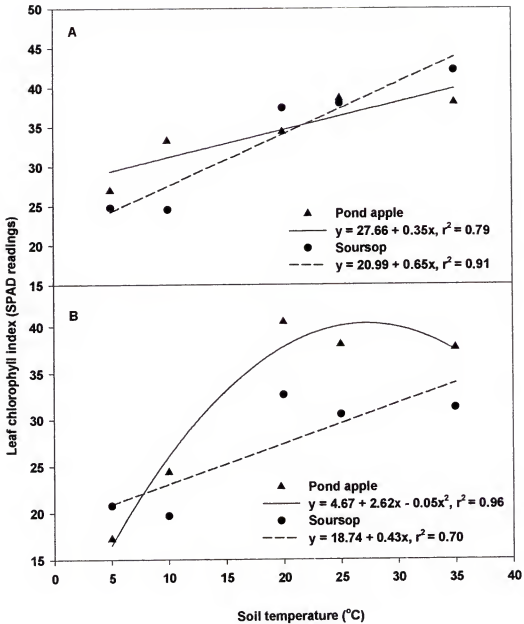


Figure 4-3. Soil temperatures and leaf chlorophyll index of (A) non-flooded and (B) flooded *Annona* species 4 weeks after flooding and soil temperature treatments were initiated. Symbols represent the means of 4 replications (block) with 6 leaf samples per plant.

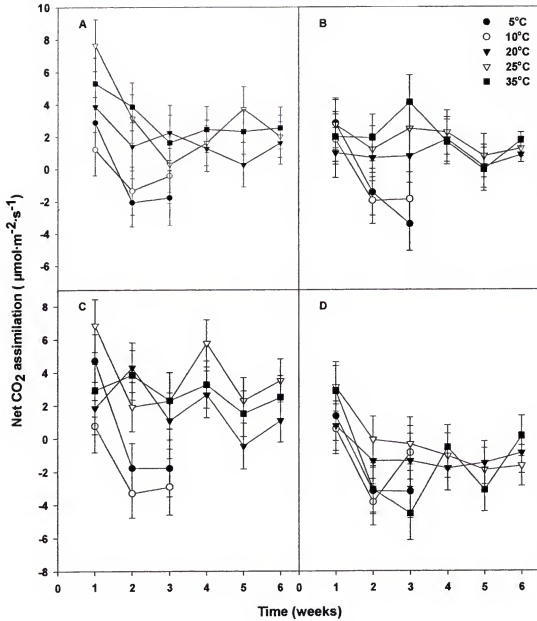


Figure 4-4. Soil temperatures and net CO₂ assimilation of (A) non-flooded pond apple, (B) non-flooded soursop, (C) flooded pond apple, and (D) flooded soursop 6 weeks after flooding and temperature treatments were initiated. Symbols represent means \pm SE of 4 replications (block). Plants at 5 and 10°C were wilted by week 4.

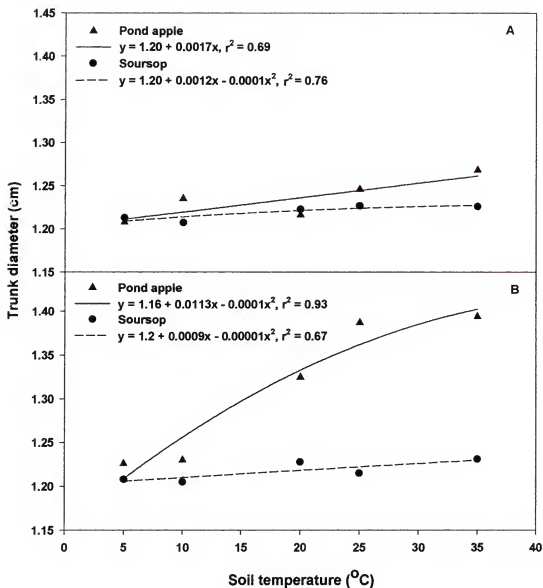


Figure 4-5. Soil temperatures and trunk diameter of (A) non-flooded and (B) flooded *Annona* species 3 weeks after flooding and temperature treatments were initiated. Symbols represent the means of 4 replications (block).

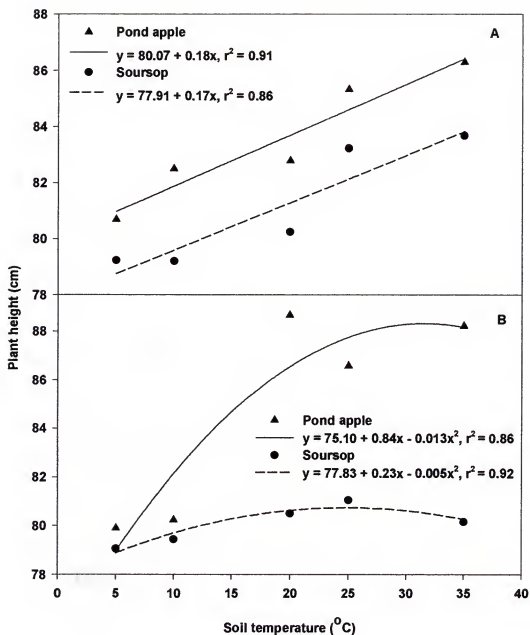


Figure 4-6. Soil temperatures and plant height of (A) non-flooded and (B) flooded *Annona* species 4 weeks after flooding and temperature treatments were initiated. Symbols represent the means of 4 replications (block).

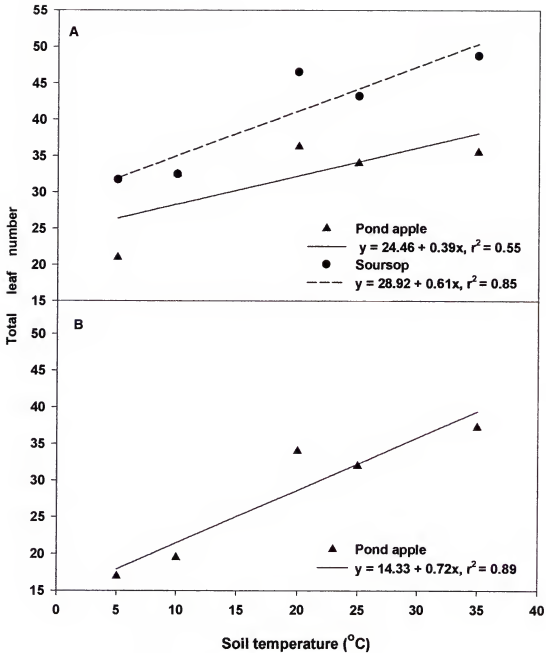


Figure 4-7. Soil temperatures and total leaf number of (A) non-flooded and (B) flooded *Annona* species 4 weeks after flooding and temperature treatments were initiated. Symbols represent the means of 4 replications (block). There were no significant differences in the total leaf number for flooded sour sop plants (data not shown).

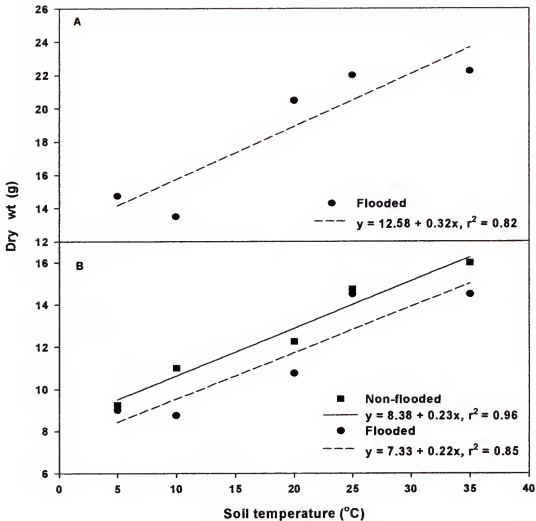


Figure 4-8. Soil temperatures and dry weights of (A) stem and (B) root of non-flooded and flooded pond apple 6 weeks after flooding and temperature treatments were initiated. Symbols represent the means of 4 replications (block). Stem dry weight of non-flooded pond apple plants and root and stem dry weights of non-flooded and flooded soursop plants were not significantly different, $P < 0.05$ (data not shown).

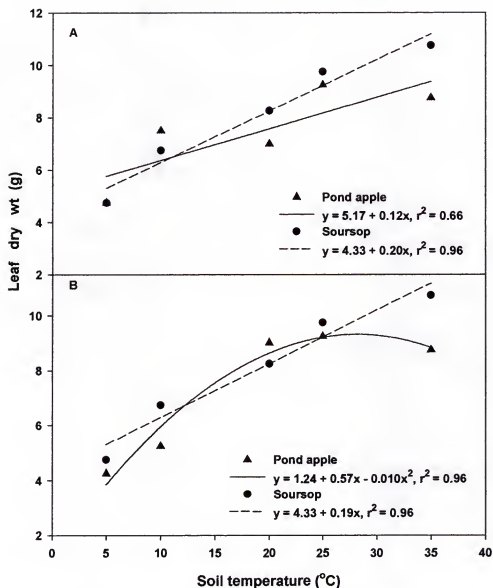


Figure 4-9. Soil temperatures and leaf dry weight of (A) non-flooded and (B) flooded *Annona* species 6 weeks after flooding and temperature treatments were initiated. Symbols represent the means of 4 replications (block).

CHAPTER 5
IRON NUTRITION, FLOODING, AND GROWTH OF POND APPLE
(*Annona glabra* L.)

Introduction

In some areas, such as south Florida, annonas are commercially grown in calcareous soils characterized by high pH (7.5 to 8.5), a high bicarbonate (HCO_3^-) concentration, and generally with a low organic matter content (Lucena, 2000). These soil conditions promote Fe deficiency for many fruit crops (Korcak, 1987), and correcting this problem is a major production cost. Additionally, there are low-lying areas with this soil type that are prone to flooding.

In alkaline soils, flooding decreases soil pH (Larson et al., 1991c). Under flooded conditions, elements such as Fe, Mg, and Mn become more soluble (Ponnamperuma, 1984; Larson et al., 1991c). Iron in the form of Fe^{3+} is reduced to Fe^{2+} , a form that is more available to plants (Ponnamperuma, 1984; Larson et al., 1991c, 1992; De Mello et al., 1998; Zude-Sasse and Lüdders, 2000; Zude-Sasse and Schaffer, 2000). Increased solubility of Fe is often one of the most important chemical changes that occurs in flooded calcareous soils (Larson et al., 1991c). Thus, annual cyclical flooding in calcareous soils may result in increased micronutrient availability and improved plant nutritional status (Laan et al., 1989; Zude and Lüdders, 2000).

Pond apple (*Annona glabra* L.) is a perennial woody tree species native to tropical and subtropical wetlands of the Americas, including wetlands of south Florida (Morton, 1987). It is generally not considered a commercial tree fruit species, although it has been

occasionally used in the production of jams and jellies (Morton, 1987). Pond apple is extremely flood tolerant (Zotz et al., 1997) and the use of pond apple as a rootstock may allow commercial production of flood-sensitive *Annona* species (Núñez-Elisea et al., 1998, 1999) in flood prone areas.

Pond apple growing in calcareous soils under non-flooded conditions exhibits Fe deficiency symptoms, suggesting that pond apple may require considerably more Fe than traditional annona rootstocks unless the soil is periodically flooded (B. Schaffer, personal communication). The most effective and common method to correct Fe deficiency in calcareous soils is soil drench applications of ferric chelated iron sources (i.e., Sequestrene-138, Fe-EDDHA) since ferrous sulfate can precipitate in calcareous soils. The commercial Fe rate for *Annona* species in calcareous soils is 2.5 g Sequestrene-138/plant (J. Crane, personal communication), but it is an expensive Fe source. Although Fe chelation is required in alkaline agricultural soils, the periodic flooding that occurs in the natural habitat of pond apple presumably results in increased Fe solubility and availability (Larson et al., 1991c). Thus, in periodically flooded soils it may be possible to use lower rates of chelated Fe than are currently used for commercial *Annona* species or a non-chelated Fe source may result in adequate Fe availability for the plant.

The objective of this study was to quantify the Fe requirement of pond apple in flooded and non-flooded calcareous soils.

Materials and Methods

The study was conducted from August to November 2000 in a sunlit glasshouse in Gainesville, Fla. Average day/night air temperatures during the experimental period ranged from 36/27 to 25/20°C, and relative humidity was 80 to 85%. Air temperature and

relative humidity were monitored and recorded using a Hobo H8 Pro Series temperature logger (Onset Computer Corporation, Pocasset, Mass.).

Plant Material

Seedling pond apple trees that were 1.5 years-old were used in the study. Trees were grown in 7.6-L containers in Krome very gravelly loam soil (loamy-skeletal, carbonatic, hyperthermic Lithic Rendoll), which is native to southern Florida (Noble et al., 1996). Plants were irrigated daily to container capacity, and fertilized (top dressing) at the beginning of the study with 15 g/plant of granulated fertilizer (10N-4.4P-8.3K, with ammonium and urea as N sources).

Treatments

Six weeks prior to treatment initiation, all plants were pruned to produce new growth flushes. After pruning, 100 uniform trees were selected and 50 were flooded and 50 remained not flooded (non-flooded). Treatments were arranged as a 2 (flooded and non-flooded) x 2 (Fe sources: chelated and non-chelated) x 5 (Fe rate) factorial. The Fe sources were Sequestrene-138 (Fe chelate containing 6% Fe, Fe-EDDHA), and ferrous sulfate (FeSO_4 , containing 20% Fe). The five Fe rates used were 0, 0.625, 1.25, 2.5, and 5.0 g/plant. Iron rates and formulations were based on those applied to commercial atemoya (*A. squamosa* L. x *A. cherimola* Mill.) trees in south Florida (2.5 g Sequestrene-138/tree, J.H. Crane, personal communication). The Fe rates of FeSO_4 were also based on the commercial amount of 2.5 g Fe/plant but were adjusted according the concentration of 20% Fe in ferrous sulfate. Iron treatments were applied as a soil drench. There were five-single tree replications for each treatment combination. Plants were subjected to the treatments for 12 weeks using the onset of leaf chlorosis of plants in the 0 g Fe/plant treatment as indicator of when to terminate the experiment.

Soil Measurements

For flooded plants, soil redox potential (Eh) was monitored using a platinum combination electrode (Ag^+/AgCl , Accumet, Fisher Scientific, Pittsburgh, Pa.) attached to a portable pH meter (Accumet AP62, Fisher Scientific, Pittsburgh, Pa.). Soil Eh was recorded 1, 3, 7, and 14 d after flooding treatments were initiated.

Leaf Nutrient Concentrations

Nutrient element concentrations were determined by sampling leaves between the 4th and 6th nodes below the shoot apex at the beginning of experiment. At the end of the experiment, macro and microelement concentrations were determined by sampling leaves between the 4th and 6th node below the shoot apex (young leaves), and leaves from below the 7th node (mature leaves), respectively. Six to seven leaves per plant-treatment combination were sampled. Leaves were gently rinsed with deionized water for about 1 min. Samples were then oven-dried at 70°C for 48 h. The dry samples were ground through a 40-mesh screen in a Wiley mill. The samples were prepared and processed for analysis using the wet ash (TKN) and dry ash procedures (Hanlon et al., 1994).

Total Kjeldahl nitrogen (TKN) concentration

Approximately 0.1 g of ground sample was transferred to a 50 ml digestion tube containing ~ 2.0 g of Kjeldahl mixture (10 g K_2SO_4 : 3g CuSO_4), and 2.5 ml of sulfuric acid. Tubes were covered with glass funnels and placed in an aluminum block digester at 380°C for 8 to 10 h. After the tubes were allowed to cool overnight, the glass funnels covering the tubs were rinsed thoroughly into the tubes with 5 to 10 ml of deionized water. The tube was shaken in a vortex mixer and the volume brought to 50 ml with deionized water. Subsamples were filtered through Whatman P8 paper. Subsamples were transferred to 20 ml polyethylene scintillation vials for analysis. Nitrogen concentration

was expressed as % dry weight (dry wt). Samples were analyzed for Total Kjeldahl Nitrogen (TKN) concentration using an Air-Segmented, Continuous-Flow, Automated Spectrophotometer.

Leaf concentrations of other nutrients

The concentrations of P, K, Mg, Ca, Fe, Mn, Zn, and Cu were determined using the dry ash procedure. Approximately 0.5 g of ground plant tissue was transferred to a 10 ml beaker and placed in a muffle furnace at 500°C for 10 to 12 h. After the sample was cooled, a few drops of 1N HCl were added to the ash, the samples were rinsed with 1 N HCl, transferred to a volumetric flask and brought to a volume of 50 ml. The samples were mixed thoroughly, and then filtered through Whatman Q8 filter paper. Subsamples were transferred to 20 ml polyethylene scintillation vials for analysis. For each treatment, concentrations of P, K, Mg, and Ca were expressed as % dry wt, and concentrations of Fe, Mn, Zn, and Cu were expressed as $\text{mg}\cdot\text{kg}^{-1}$ dry wt. Concentrations of P, K, Mg, Ca, Fe, Mn, Zn, and Cu were determined using an Inductively Coupled Argon Plasma Spectrometer (Spectro-CIROS CCD, FTCEA000, Germany) at the IFAS Analytical Research Laboratory, University of Florida, Gainesville, Fla.

Leaf Chlorophyll and Growth Measurements

Leaf chlorophyll index was determined using a chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd, Japan). Measurements were made on six leaves located between the 4th and 6th nodes of the shoot apex (young leaves) and six leaves below the 7th node (mature leaves) from treatment initiation (day 1) and at weekly intervals thereafter. Data were expressed as SPAD readings (Schaper and Chacko, 1991). Axillary shoot length was determined by measuring the length of one tagged shoot per plant from

the leaf axil to the apical bud. Shoots were measured from the beginning (day 1) of the study and then at weekly intervals.

Statistical Analysis

The treatments were arranged in a completely randomized design (CRD) with five single-plant replications for each treatment combination. Data were analyzed by a combination of statistical tests, including analysis of variance and multiple comparison (LSMEANS), repeated measures, and covariance procedures using SAS (SAS Institute, Cary, N.C.) statistical software. Regression analysis was done using SigmaPlot program (SPSS Science, Chicago, Ill).

Results and Discussion

Soil Redox Potential (Eh)

There was no interaction between rate or formulation Fe and flooding treatments for soil Eh ($P < 0.05$); therefore all flooding treatments were pooled for reporting Eh values. The soil became anaerobic ($Eh \leq 200$ mV) (Ponnamperuma, 1984) within 3 d after flooding (Fig. 5-1). Soil Eh decreased to -158 mV by the 7th d of flooding. Thereafter, soil Eh changed little reaching -163 mV on day 14. Thus, plant roots in the flooded treatment were under reduced soil conditions (low soil Eh), and therefore restricted availability of O₂ from day 3 through week 12. Núñez-Elisea et al. (1999) found similar results for *Annona* species in flooded Krome very gravely loam soil. The rapid decrease of soil Eh within 1 week may be attributed to high air temperatures, which averaged 29.6°C during the first week of the study. As temperatures increase, root respiration and microbial activity in the soil increase causing rapid O₂ depletion and reduction of soil Eh (Larson et al., 1992).

Morphological Adaptations to Flooding

Some plants can adapt to flooding stress either by avoiding oxygen deficits by developing aerenchyma, adventitious roots or hypertrophied (swollen) stem lenticels for improved root gas exchange, or by physiologically adapting to oxygen deficits (Schaffer et al., 1992; Armstrong et al., 1994; Crawford and Braendle, 1996). In this study, pond apple developed hypertrophied (swollen) trunk lenticels within 3 d of flooding. Pond apple also produced adventitious roots, upward root growth through the soil from preexisting roots, and basal trunk swelling. These morphological changes in response to flooding, reported previously in pond apple (Núñez-Elisea et al. 1998, 1999), were presumably adaptations to improve flood-tolerance since there was 100% plant survival after 12 weeks of flooding. Hypertrophied (swollen) trunk lenticels may facilitate internal oxygen diffusion to flooded roots (Armstrong, 1968; Kozłowski, 1984; Jackson and Attwood, 1996) and/or function as excretory sites for potentially toxic metabolites formed in the roots during anaerobic respiration (Chirkova and Gutman, 1972). Similarly, basal trunk swelling is thought to help gas diffusion because there is an increased porosity among stem tissues, thus enhancing internal aeration (Armstrong et al., 1994; Yamamoto et al., 1995). Adventitious roots may help to maintain nutrient and water absorption under flooded conditions (Sena Gomes and Kozłowski, 1980).

Leaf Nutrient Concentrations

After flooding treatments were imposed, significant interactions ($P \leq 0.05$) were observed between Fe fertilization and flooding treatments for leaf nutrient concentration, leaf chlorophyll index, and shoot length. Therefore, the effect of Fe fertilization treatments on all variables is reported separately for each flooding treatment at the last measurement date (week 12).

At the beginning of the study, before applying Fe and flooding treatments, means and standard errors (n=100 plants) for N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu concentrations in young leaves were $1.28 \pm 0.141 \%$, $0.26 \pm 0.014 \%$, $2.06 \pm 0.056 \%$, $2.31 \pm 0.205 \%$, and $0.50 \pm 0.021 \%$, $51.80 \pm 5.83 \text{ mg}\cdot\text{kg}^{-1}$ dry wt, $50.44 \pm 12.57 \text{ mg}\cdot\text{kg}^{-1}$ dry wt, $43.52 \pm 6.62 \text{ mg}\cdot\text{kg}^{-1}$ dry wt, and $14.05 \pm 2.76 \text{ mg}\cdot\text{kg}^{-1}$ dry wt, respectively.

Macronutrients in young leaves

Twelve weeks after treatments were initiated, all macronutrients in young leaves were significantly affected by flooding and Fe treatments (Appendix A).

Leaf N concentration was generally lower for flooded than for non-flooded plants (Table 5-1). This response may be due to denitrification (Ponnamperuma, 1984; Olde et al., 2002), which occurs quickly because NO_3^- is the first electron acceptor to be reduced in anaerobic soil following O_2 depletion (Reddy and Patrick, 1983). The critical redox potential at which NO_3^- is reduced to N_2 is 200 mV (Patrick and Jugsujinda, 1992), and in this study soil Eh was < 200 mV within 3 d of flooding. Similarly, Larson et al. (1991c) reported a rapid decrease in NO_3^- concentration one week after Krome very gravelly loam soil was flooded.

In general, leaf N concentrations were lower than those reported as the sufficiency range for other *Annona* species such as atemoya (*Annona squamosa* L. x *A. cherimola* Mill) (George et al., 1987). For atemoya, George et al. (1989) reported a general decline in the leaf N concentration throughout season, which was influenced by vegetative, flushing, suggesting a high demand for N to sustain growth compared to the other leaf nutrients. Similarly, Gettys and Sutton (1999) found that 6-months-old pond apple seedlings in containers had the greatest growth after application of the highest N rate of

14.96 g N compared to application of 0.94 to 14.96 g N/plant. Thus, it is possible that the 1.5 g N/plant applied in this study was not sufficient to sustain the rapid growth of pond apple (Núñez-Elisea et al., 1999).

For plants in the chelated, flooded treatments, leaf N concentration tended to be the highest at Fe rates of 0.625 to 1.25 g/plant. There was no effect of flooding on foliar N concentrations in the non-chelated treatments, regardless of Fe rates (Table 5-1).

Flooded plants generally had lower leaf P concentrations than non-flooded plants (Table 5-1). Leaf P concentrations of flooded plants were lower than those reported for optimal growth of atemoya (George et al., 1987). In alkaline soils where P is not very soluble, flooding can increase P availability due to a reduction in insoluble P compounds (De Mello et al., 1998). However, Ca can also be dissolved and react with P to form insoluble complexes (Ponnamperuma, 1984), causing a decreased foliar concentration of P, as observed in flooded plants of this study. The Krome very gravelly loam soil used in this study has high Ca because it is derived from limestone (CaCO_3) parent material (Noble et al., 1996). Additionally, a decreased leaf P concentration may be due to a dilution effect in the soil solution (Ponnamperuma, 1984). These results are consistent with studies of flooded mango (Larson et al., 1992), and nuttall oaks (Pezeshki et al., 1999) where flooding reduced leaf P concentration as well.

For plants in the non-chelated Fe treatments, there was no effect of flooding on foliar P concentrations, but for chelated treatments an Fe rate of 5 g/plant tended to decrease foliar P concentration (Table 5-1). Flooding results in an increased soil Fe concentration, which may also interfere with P uptake causing P deficiency (Kozlowski and Pallardy, 1984). For plants in chelated, non-flooded treatments, there was no effect of

Fe application rate on leaf P concentrations. In general, non-chelated, non-flooded treatments exhibited decreased leaf P concentration as Fe rates increased.

For plants fertilized with either chelated or non-chelated Fe, flooding resulted in lower foliar K concentrations compared to non-flooded plants (Table 5-1). Leaf K concentrations in flooded plants were lower than values reported as the critical range for optimal growth of atemoya (George et al., 1987). Larson et al. (1992) and Pezeshki et al. (1999) also found that flooding reduced leaf K concentrations in mango and nuttall oak, respectively. Flooding generally inhibits K^+ uptake and decreases leaf K concentration (Kozłowski and Pallardy, 1984). The rate of K^+ absorption is directly related to the ATP content of the root cells (Shuman, 1994), and it is well known that flooding reduces root respiration (Schaffer et al., 1992), and thus production of ATP.

For plants in the chelated, non-flooded treatments, the highest leaf K concentration occurred in plants receiving no Fe (Table 5-1). The reduced plant growth rate and thus low demand for K (May et al., 1994), because of Fe deficiency, may have resulted in K accumulation in the leaf. In non-flooded conditions, Fe deficiency generally increases leaf K concentration and decreases leaf Ca concentration, and consequently causes a marked increase in the K/Ca ratio (Abadía et al., 1985; Belkhodja et al., 1998). For plants in the non-chelated, flooded treatments, leaf K concentration increased as Fe rates increased. There was no effect of Fe rate on leaf K concentration of plants in non-chelated, non-flooded treatments (Table 5-1).

Except for the plants in non-chelated, non-flooded treatments, leaf K concentrations in this study were lower than the sufficiency range for atemoya (George et al., 1987). This study was conducted from August to November, and *Annona* species under

subtropical conditions are semi-deciduous plants from early winter to spring. Thus, the low leaf K concentrations may be associated with the physiological age of the leaves. Mondal and Chattopadhyay (1993) also reported that leaf K concentration of sugar apple (*A. squamosa* L.) decreased from August to November in India, which they related to leaf age.

Foliar Ca concentrations were higher for plants in all flooding and Fe treatments than those reported for optimum growth of atemoya (George et al., 1987). High leaf Ca concentrations may have been a result of the high Ca concentration in the soil solution since Krome very gravelly loam soil is derived from limestone (CaCO_3) parent material (Noble et al., 1996), and the high amount of Ca in plants is a result of high levels of Ca in the soil solution rather than to efficiency of uptake (Shuman, 1994).

In general, plants in the chelated treatments had higher leaf Ca concentration than plants in non-chelated treatments (data not shown). For plants in chelated, flooded treatments, leaf Ca concentration decreased as Fe application rate increased. Leaf Ca concentrations were higher in plants in the chelated, non-flooded treatments than in chelated, flooded treatments (Table 5-1). Although flooding can increase Ca^{2+} concentration in alkaline soil, P can form insoluble complexes with Ca, making Ca less available to the plant (Ponnamperuma, 1984). Larson et al. (1992) also observed lower foliar Ca concentrations for flooded mango plants that received chelated Fe in calcareous soil than those of non-flooded plants.

For plants in the chelated, non-flooded treatments, foliar Ca concentration tended to decrease as Fe application rates increased (Table 5-1). In non-flooded conditions, Fe deficiency generally decreases leaf Ca concentration (Abadía et al., 1985; Belkhdja et

al., 1998). For plants in the non-chelated, flooded treatments, the lowest leaf Ca concentrations were observed at Fe application rates of 0.625 and 2.5 g/plant. There was no effect of Fe application rates on leaf Ca concentration of plants in the non-chelated, non-flooded treatments.

In general, plants in the chelated treatments had a higher leaf Mg concentration than plants in the non-chelated treatments (data not shown). There was no effect of flooding on leaf Mg concentrations among Fe rates in either the chelated or the non-chelated treatments (Table 5-1). In flooded alkaline soils, MgCO_3 can be dissolved and the increased concentration of reduced cations (Fe^{2+} , Mn^{2+}) in the soil solution can lead to displacement of Mg^{2+} from the exchange complex, thereby increasing its availability (Reddy and Patrick, 1983; Larson et al., 1991c). However, flooded plants tended to have lower leaf Mg concentrations than non-flooded plants, especially for the chelated treatments (Table 5-1). This response may be due to a dilution of Mg in the soil solution (Ponnamperuma, 1984).

For both flooding treatments, plants receiving non-chelated Fe tended to have a lower leaf Mg concentration than those reported as critical for optimal growth of atemoya (George et al., 1987). For plants in the chelated, non-flooded treatments, leaf Mg concentration decreased as the Fe application rate increased, with the lowest values occurring at 2.5 and 5 g Fe/plant. For plants in non-chelated, non-flooded treatments, leaf Mg concentration generally increased as Fe application rates increased. These responses may be associated with the leaf Ca concentration observed in this study (Table 5-1) because in soils with high pH, Mg uptake is more related to the ratio of Mg/Ca rather than soil Mg concentration alone (Shuman, 1994).

Micronutrients in young leaves

In general at the end of study, regardless of flooding treatment, leaf Fe concentration was higher for plants in chelated Fe than those in non-chelated Fe treatment (Fig. 5-2). These results agree with other studies with grape and peach in calcareous soil (Reed et al., 1988). Chelated Fe is more readily available for plant uptake in alkaline soils than non-chelated Fe. Chelated Fe fertilizers, such as Sequestrene-138 (Fe-EDDHA), are synthetic compounds that bond or complex Fe and are highly water soluble. The “complexation” protects Fe from the usual soil reactions avoiding formation of insoluble $\text{Fe}(\text{OH})_3$ (Chen and Barak, 1982). Thus, more soluble Fe is available in the soil solution for plant uptake, including the calcareous soils used in this study.

By week 12, there was a quadratic relationship between Fe application rate and leaf Fe concentration for plants in the chelated, flooded treatments ($r^2 = 0.55$), with the maximum value ($65 \text{ mg} \cdot \text{kg}^{-1}$ dry wt) at 2.5 g Fe/plant (Fig. 5-2A). Leaf Fe concentration was lowest at an Fe application rate of 5 g/plant . For plants in the chelated, non-flooded treatments, leaf Fe concentration increased from 0 to 5 g Fe/plant , although the relationship between Fe rate and leaf Fe concentration was weaker ($r^2 = 0.37$) than for plants in the chelated, flooded treatments (Fig. 5-2A). The lower Fe rate required by the flooded plants (2.5 g/plant) compared to the non-flooded plants (5 g/plant) may be due to an increased concentration of soluble Fe in the flooded soil (Ponnamperuma, 1984; Larson et al., 1991c; De Mello et al., 1998). Under anaerobic conditions, Fe^{3+} is reduced to Fe^{2+} (Ponnamperuma, 1984) increasing solubility of Fe (Larson et al., 1991c). The critical redox potential at which Fe^{3+} is reduced to Fe^{2+} is 100 mV (Patrick and Jugsujinda, 1992). In this study, soil Eh reached -21 mV within 3 d of flooding.

There were quadratic relationships between Fe application rate and leaf Fe concentration with $r^2 = 0.71$ and $r^2 = 0.61$, respectively for flooded and non-flooded plants fertilized with non-chelated Fe (Fig. 5-2B). For plants in the non-chelated, non-flooded treatments, the maximum leaf Fe concentration was observed at 5 g Fe/plant. The non-chelated Fe source was presumably too insoluble in calcareous soils for adequate plant uptake, thus only high Fe application rates increased leaf Fe concentration. In non-flooded plants fertilized with non-chelated Fe, Fe application rates between 0.625 to 2.5 g/plant resulted in lower leaf Fe concentrations than those reported as the critical ranges for atemoya (George et al., 1987). For plants in the non-chelated treatments at Fe application rates of 0.625 and 1.25 g/plant, flooding resulted in higher leaf Fe concentrations than in non-flooded plants that received the same amount of Fe. This response may also have been due to flooding-induced increases in soil Fe^{2+} concentrations.

Twelve weeks after treatments were initiated, Mn, Zn, and Cu concentrations in young leaves were significantly affected by flooding and Fe treatments (Appendix B).

For plants in chelated or non-chelated Fe treatments, Mn concentrations were greater in leaves of flooded than in those of non-flooded plants (Table 5-2). When soil is flooded Mn^{4+} is reduced to Mn^{2+} , which is more available in the soil solution (Ponnamperuma, 1984). The critical redox potential at which Mn^{4+} is reduced to Mn^{2+} is approximately 200 mV (Patrick and Jugsujinda, 1992), which is above the soil Eh (-21 mV) reached within 3 d of flooding in this study. The observed increase in foliar Mn concentration in flooded trees is consistent with observations of mango in calcareous soil (Larson et al., 1992) as result of solubilization of soil Mn by flooding (Larson et al.,

1991c). However, in general, leaf Mn concentrations for all flooded and Fe treatments tended to be lower than those reported as critical values for atemoya (30-90 mg·kg⁻¹ dry wt; George et al., 1987), especially in non-flooded plants, although, no visual Mn deficiency symptoms were observed.

In flooded plants, leaf Mn concentrations were lower than critical values (George et al., 1987), although flooding can increase soil Mn concentration. This may be due to the higher relative solubility of Mn²⁺ than Fe²⁺ in reduced soils. Thus, Mn²⁺ is usually depleted before Fe²⁺ with a corresponding increase in the Fe/Mn ratio in the soil. Therefore, uptake of Mn could be decreased by high levels of available Fe (Reddy and Patrick, 1983).

For plants in the chelated, flooded and chelated, non-flooded treatments, the leaf Mn concentration tended to be the lowest at an Fe application rate of 5 g Fe/plant (Table 5-2). For flooded and non-flooded plants with non-chelated Fe, there was no effect of Fe application rate on leaf Mn concentrations (Table 5-2).

For plants in both the chelated and non-chelated treatments, flooding decreased leaf Zn concentrations (Table 5-2). Flooding can decrease pH in alkaline soil, and thus Zn availability may increase (Kozłowski and Pallardy, 1984). However, prolonged flooding can reduce the mobility of some cations such as Zn and Cu because these metals can precipitate with sulfides under reduced conditions (Engler and Patrick, 1975). A negative correlation between Fe and Zn has been reported for mandarin (Saatçi and Ya Mur, 2000), sour orange and bitter almond (Shibli et al., 2002). The leaf Zn concentrations observed in this study were in the critical range reported for optimal growth of atemoya (George et al., 1987). For plants in chelated, flooded treatments, the leaf Zn concentration

tended to be highest between 0.625 to 2.5 g Fe/plant, and for chelated, non-flooded treatments at 0 g Fe/plant.

Although there was no significant effect of flooding on foliar Cu concentration regardless of Fe application rate, flooded plants tended to have lower leaf Cu concentrations than non-flooded plants (Table 5-2). Some metallic cations, such as Cu can precipitate with sulfides under anoxic conditions (Engler and Patrick, 1975). For plants in the chelated, non-flooded treatment, in general leaf Cu concentration decreased as the Fe application rate increased (Table 5-2). These results are consistent with the negative correlation found between Fe and Cu in sour orange and bitter almond (Shibli et al., 2002). In general, foliar Cu concentrations in this study were in the critical range reported for atemoya (George et al., 1987).

Flooding also increased Fe and Mn concentrations in mature leaves (data not shown).

Leaf Chlorophyll Index

Leaf chlorophyll index generally increased throughout the study (12 weeks) as Fe application rates increased, regardless of Fe sources or flooding treatments (data not shown). Young leaves of plants in the chelated treatments usually had greater leaf chlorophyll index than plants in the non-chelated treatments (Fig. 5-3). Ferric-EDDHA (Sequestrene-138) has a high stability compared to other chelated Fe sources (Brown, 1978). That characteristic permits Fe-EDDHA to complex and tightly holds Fe, increasing its solubility and availability to plant roots. Therefore, increased availability and uptake of Fe increase leaf chlorophyll concentration because nearly all of a plant's Fe is in the chloroplast (Miller et al., 1995). Additionally, Fe is involved in chlorophyll synthesis (Terry and Abadía, 1986). In contrast, Larson et al. (1992) did not find an effect

of flooding on leaf chlorophyll concentration in mango plants treated with 5 g of chelated Fe in Krome very gravelly loam soil because leaf chlorophyll concentration was more related to reduced leaf Mn concentration than increased leaf Fe concentration.

Twelve weeks after treatments were initiated for flooded and non-flooded plants receiving chelated Fe, there were quadratic relationships between Fe application rate and leaf chlorophyll index (Fig. 5-3A), although the relationship was weaker for non-flooded than flooded plants. For plants in the chelated, flooded treatments, the highest leaf chlorophyll index occurred at Fe application rates between 1.25 and 2.5 g Fe/plant, and the lowest occurred at 5 g Fe/plant. For plants in chelated, non-flooded treatments the maximum leaf chlorophyll index was between 2.5 and 5 g Fe/plant. In general, plants in the chelated, flooded treatments had a lower leaf chlorophyll index than non-flooded plants. This response may be more associated with the lower leaf Mg and Cu concentrations observed for plants in the chelated, flooded treatment than for plants in the chelated, non-flooded treatment (Table 5-1 and 5-2), since Mg is one of the major constituents of the chlorophyll molecule. Copper may also play a role in the synthesis of chlorophyll (Shuman, 1994).

For plants in non-chelated, flooded and non-chelated, non-flooded treatments, there were quadratic relationships between the Fe application rate and leaf chlorophyll index with the highest value at an Fe application rate of 5 g Fe/plant for the non-chelated, flooded treatment and 2.5 g Fe/plant for the non-chelated, non-flooded treatment (Fig. 5-3B). In general, flooded plants had higher leaf chlorophyll indices than non-flooded plants. This increase may be due to flood-induced increases in leaf Fe concentration observed for plants in the non-chelated, flooded treatments (Fig. 5-2B).

Leaf chlorophyll index of mature leaves was higher for plants in the chelated than in the non-chelated treatments, but there was no effect of flooding on the leaf chlorophyll index (data not shown).

Shoot Length

In general, the shoot length was greater for plants in chelated Fe treatments than for plants in non-chelated Fe treatments (Fig. 5-4). Increased availability of Fe in chelated soils and subsequent higher chlorophyll concentration can result in increased net CO₂ assimilation (*A*) and a subsequent increase in shoot growth. Net CO₂ assimilation in pond apple follows the same as leaf chlorophyll patterns (see Chapters 3 and 4). Additionally, Fe is also a constituent of ribonucleotide reductases (RNR) (Nordlund et al., 1990; Reichard, 1993), which produce the deoxyribonucleotides needed for DNA synthesis and thus meristematic growth.

By week 12 for flooded and non-flooded plants in the chelated treatments, there were quadratic relationships between the Fe application rate and shoot length (Fig. 5-4A). For plants in the chelated, flooded treatment, the greatest shoot growth occurred at 2.5 g Fe/plant. For plants in chelated, non-flooded treatment the greatest shoot length was observed between 2.5 and 5 g Fe/plant.

For plants in the chelated treatments, leaf Fe concentrations of flooded plants were higher than those of non-flooded plants (Fig 5-2A). However, the leaf chlorophyll index (Fig. 5-3A) and shoot growth (Fig. 5-4A) were lower in flooded plants than in non-flooded plants. This response suggests that although plants in chelated, flooded treatments potentially had an increased availability of Fe²⁺, Fe may be confined to the leaf apoplast (Mengel, 1994) and be poorly utilized. Pond apple has a low leaf ferric chelate reductase (FCR) activity (see Chapter 7). This low leaf FCR activity may

decrease Fe^{3+} reduction by mesophyll cells and consequently depress Fe^{2+} transport across the leaf plasmalemma resulting in a decreased leaf chlorophyll content as observed in plants in chelated, flooded treatment.

For plants in the non-chelated treatments, shoot length was generally higher in flooded plants than in non-flooded plants (Fig. 5-4B), which followed the same leaf chlorophyll patterns (Fig. 5-3B). The adventitious roots that pond apple developed in response to flooding may partially explain the apparent continued nutrient and water absorption to sustain plant growth under flooded conditions (Sena Gomes and Kozlowski, 1980). The development of hypertrophied trunk stem lenticels may facilitate oxygen diffusion to flooded roots (Kozlowski, 1984) and produce energy to maintain the ion uptake.

For plants in the non-chelated, flooded treatment, the greatest shoot length was observed at an Fe application rate of 5 g Fe/plant (Fig. 5-4B), and for the non-chelated, non-flooded treatments at 2.5 and 5 g Fe/plant. Even though plants in the non-chelated, non-flooded treatment had high leaf concentrations of N, P, K, and Cu, they had less shoot growth than flooded plants. Increases in leaf nutrient concentrations concomitant with increased Fe chlorosis may be a result of reduced leaf expansion as a result of Fe stress leading to a relative increase in nutrient concentrations (Römheld, 2000). In this study, leaves of plants in non-chelated, non-flooded treatment were smaller than those in the other treatments.

Conclusions

Morphological changes in pond apple in response to flooding may allow plants to tolerate anaerobic soil conditions as evidenced by 100% plant survival after 12 weeks of flooding. Flooding decreased leaf concentration of N, P, K, Ca, Mg, Zn, and Cu, and

increased Fe and Mn in pond apple plants. Although Fe chelation is required in alkaline agricultural soils, the periodic flooding that occurs in some areas can result in a higher availability of Fe to plant uptake, thus improving pond apple nutritional status. Pond apple plants in the chelated, non-flooded treatments had the highest leaf chlorophyll index and growth compared to the other Fe and flooding treatments, with the optimum Fe rate at 2.5 to 5 g/plant. These results may be useful in nutrient management of commercial annona species on pond apple rootstock.

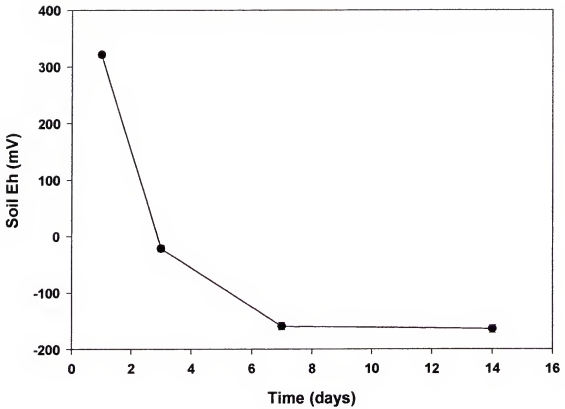


Figure 5-1 Effect of flooding on soil redox potential (Eh) of pond apple over time. Symbols represent means \pm SE of 5 Fe rates and 2 Fe sources per 5 single plant replications. SE bars not visible are masked by the symbols.

Table 5-1. Effect of Fe fertilization and flooding treatments on young leaf macronutrient concentrations of pond apple 12 weeks after treatments were initiated.

Treatments		Nutrient (% dry wt) ²					
Iron form	Flooding	Fe rate (g/plant)	N	P	K	Ca	Mg
Chelated (Fe-EDDHA)	Flooded	0	1.39b	0.13a	0.70	2.08a	0.35
		0.625	1.63ab	0.12ab	0.72	1.86ab	0.30
		1.25	1.73a	0.14a	0.63	1.77b	0.29
		2.5	1.36b	0.14a	0.83	1.66b	0.31
		5.0	1.29b	0.11b	0.60	1.57b	0.30
	Non-flooded	0	1.55	0.20	1.19a	2.52a	0.44a
		0.625	1.45	0.18	0.69b	2.79a	0.43a
		1.25	1.61	0.19	0.81b	2.49a	0.37ab
		2.5	1.79	0.17	0.79b	2.30ab	0.35b
		5.0	1.71	0.17	0.80b	1.82b	0.34b
Non-chelated (FeSO ₄)	Flooded	0	1.34	0.11	0.50bc	2.11a	0.34
		0.625	1.58	0.12	0.60bc	1.73bc	0.32
		1.25	1.53	0.14	0.65abc	1.99ab	0.33
		2.5	1.63	0.14	0.75ab	1.65c	0.29
		5.0	1.60	0.14	0.87a	2.02a	0.28
	Non-flooded	0	1.92	0.26a	1.61	1.47	0.26bc
		0.625	2.87	0.27a	1.69	1.22	0.23c
		1.25	2.38	0.18ab	1.24	1.72	0.30abc
		2.5	1.87	0.17b	1.15	1.81	0.32ab
		5.0	2.15	0.16b	0.95	2.06	0.36a

²Mean separation within columns by Fe form and flooding treatment are by LSMEANS ($P \leq 0.05$). The absence of letters indicates no significant difference among means. Values represent 5 single plant replications with 6 leaf samples per plant.

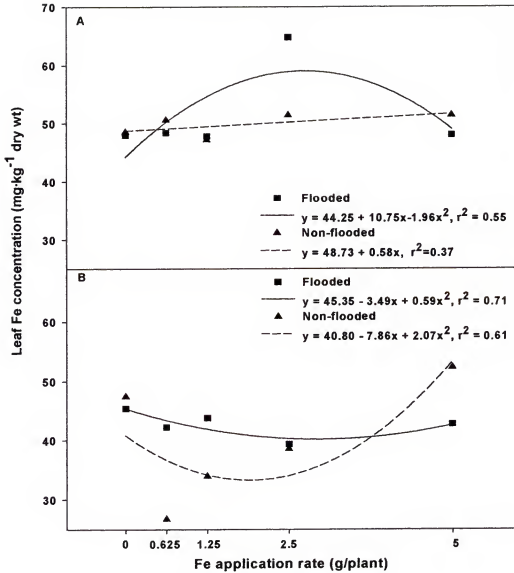


Figure 5-2. Iron application rates and young leaf Fe concentrations of flooded and non-flooded pond apple fertilized with (A) chelated Fe or (B) non-chelated Fe 12 weeks after treatments were initiated. Symbols represent the means of 5 single plant replications with 6 leaf samples per plant.

Table 5-2. Effect of Fe fertilization and flooding treatments on young leaf micronutrient concentrations of pond apple 12 weeks after treatments were initiated.

Iron form	Treatments		Nutrient ($\text{mg} \cdot \text{kg}^{-1}$ dry wt) ²		
	Flooding	Fe rate (g/plant)	Mn	Zn	Cu
Chelated (Fe-EDDHA)	Flooded	0	34.00a	19.00b	10.00
		0.625	28.8ab	22.20a	9.80
		1.25	28.40ab	21.20ab	10.20
		2.5	33.40a	22.20a	10.00
		5	26.60b	19.80ab	11.20
	Non-flooded	0	17.25a	38.25a	14.50a
		0.625	17.00a	28.20b	13.60a
		1.25	13.20ab	23.6bc	12.40ab
		2.5	13.80ab	32.00b	11.40b
		5	10.40b	23.00c	10.80b
Non-chelated (FeSO ₄)	Flooded	0	28.80	21.80	9.60
		0.625	25.25	23.25	10.25
		1.25	34.60	19.80	8.60
		2.5	26.40	19.60	8.80
		5	25.75	21.00	10.00
	Non-flooded	0	19.80	29.40	14.80
		0.625	17.25	29.00	16.00
		1.25	16.00	23.75	13.25
		2.5	16.40	28.40	12.40
		5	20.33	24.67	12.66

²Mean separation within columns by Fe form and flooding treatment are by LSMEANS ($P \leq 0.05$). The absence of letters indicates no significant difference among means. Values represent 5 single plant replications with 6 leaf samples per plant.

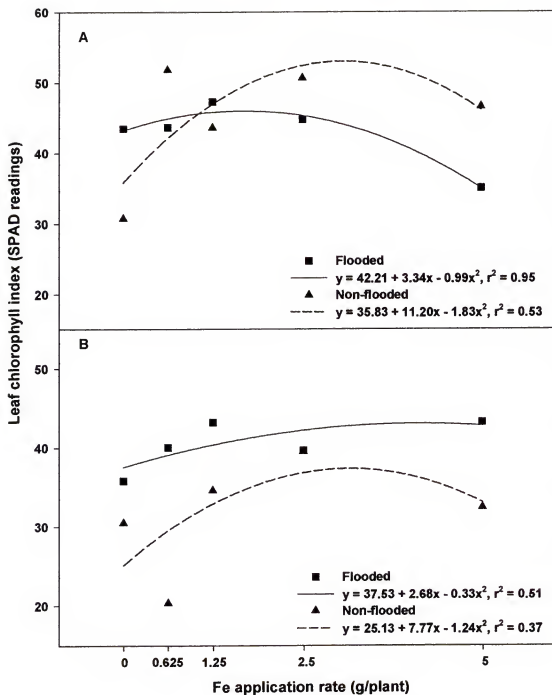


Figure 5-3. Iron application rates and young leaf chlorophyll index of flooded and non-flooded pond apple fertilized with (A) chelated Fe or (B) non-chelated Fe 12 weeks after treatments were initiated. Symbols represent the means of 5 single plant replications with 6 leaf samples per plant.

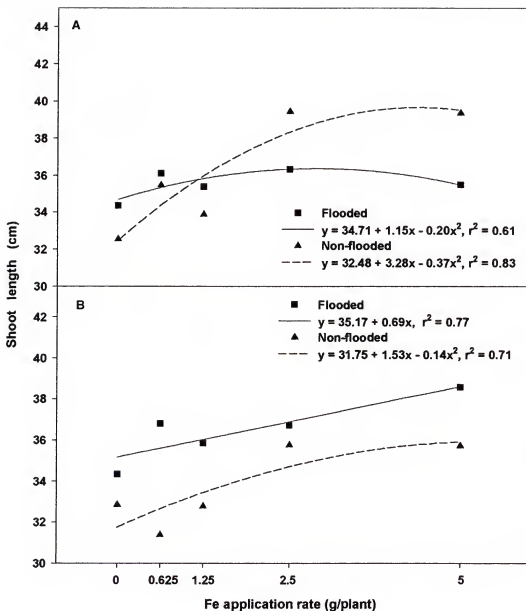


Figure 5-4. Iron application rates and shoot length of flooded and non-flooded pond apple fertilized with (A) chelated Fe or (B) non-chelated Fe 12 weeks after treatments were initiated. Symbols represent the means of 5 single plant replications.

CHAPTER 6 IRON CHELATE REDUCTASE ACTIVITY IN TWO *Annona* SPECIES AS AFFECTED BY IRON NUTRITION

Introduction

The *Annonaceae* includes several subtropical and tropical woody, perennial tree fruit species with economic importance throughout the world. Two *Annona* species, pond apple (*A. glabra* L.) and soursop (*A. muricata* L.) show promise as flood-tolerant rootstocks for commercial *Annona* species (Núñez-Elisea et al., 1999) in areas that are prone to cyclical flooding such as southern Florida. Pond apple is native to subtropical wetlands in Florida, the Caribbean, and South America, and is often found growing in wetlands, hence its common name, pond apple. Soursop is the most tropical *Annona* species, native to the Caribbean and South America (Popenoe, 1920; Nakasone and Paull, 1998). Soursop grows best in the well-drained semi-dry soils (Morton, 1987).

In some regions of the world (i.e., southern Florida), tropical fruit trees, including annonas, are grown in calcareous soils. Calcareous soils have high pH (7.5 to 8.5), high concentration of bicarbonate, and generally a low organic matter content (Lucena, 2000). A common problem in these soils is an inadequate supply of soluble iron (Fe), which results in deficiency in many crops (Korcak, 1987; Tagliavini and Rombolá, 2001). Crops with Fe deficiency display leaf chlorosis, and growth and yield suppression (Welkie, 1995; De la Guardia et al., 1995; Zouari et al., 2001).

Regulation of Fe uptake is a whole plant, shoot, or root controlled response. Therefore, plants need special mechanisms to obtain Fe from soluble forms for proper

growth, especially in neutral and alkaline soils. Plants use two different strategies to alleviate Fe deficiency: Strategy I and Strategy II (Marschner and Römheld, 1995). Strategy II plants (graminaceae species) are generally characterized by increases in the biosynthesis and secretion of compounds that are highly effective as Fe(III) chelators. These compounds are called phytosiderophores. One of the most common physiological responses of Strategy I plants (dicotyledonous and non-graminaceae monocotyledonous species) is increased activity of an inducible Fe(III) chelate reductase (FCR) enzyme in root cell plasma membranes induced by Fe stress. Induction of root FCR activity in response to Fe stress has been studied in many fruit crops, including grape (Nikolic et al., 2000; Dell'Orto et al., 2000), peach (De la Guardia et al., 1995; Alcántara et al., 2000), citrus (Manthey et al., 1993; Manthey et al., 1994; Castle and Manthey, 1998; Pestana et al., 2001), avocado (Manthey and Crowley, 1997), and papaya (Marler et al., 2002). However, some studies have reported that root FCR activity of Fe-deficient plants was lower than that in the Fe-sufficient plants (Romera et al., 1991b; Tagliavini et al., 1995; Gogorcena et al., 2000; Zouari et al., 2001).

Information is lacking concerning the tolerance of *Annona* species to low Fe levels, which is a widespread problem in calcareous soils throughout the world. The objective of this study was to compare the effect of Fe nutrition on root FCR activity in pond apple and soursop, two *Annona* species from different native habitats with potential for use as flood-tolerant commercial annona rootstocks.

Materials and Methods

The study was conducted from July-November 2001 in a sunlit glasshouse in Gainesville, Fla. Average day/night air temperatures during the experimental period ranged from 32/25 to 25/20°C, and relative humidity was 80 to 85%. Air temperature and

relative humidity were monitored using a Hobo H8 Pro Series (Onset Computer Corporation, Pocasset, Mass.) temperature logger.

Plant Material

Four-months-old seedlings of soursoy and pond apple were used. Before applying treatments, plants were fertigated monthly with 14 g/3.8 L of water-soluble fertilizer (20N-20P-20K, with ammoniacal, nitrate, and urea as N source).

Treatments

Twelve uniform trees of each species were selected, their root systems washed, and plants were grown hydroponically in 2-L plastic bottles in a nutrient solution. The plastic bottles were wrapped with aluminum foil to prevent algal growth. All plants were acclimated for 3 weeks in a complete nutrient solution, after which plants were treated with a complete nutrient solution containing 90 μM Fe(III)-DTPA (diethylenetriaminepenta-acetic acid, iron (III) disodium salt dihydrate) as the Fe source (+Fe), or a complete nutrient solution without Fe (-Fe). The solution also contained 0.5 M NaNO_3 , 0.05 M $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0045 M H_3BO_3 , 0.001 M $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.001 M $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0003 M $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The solution pH was buffered with 10 mM MES [2-(4-morpholino) – ethane sulfonic acid] at pH 6.5, and maintained at pH 6.5 by daily monitoring with a standard electrode attached to a pH meter (Accumet AP62, Fisher Scientific, Pittsburgh, Pa.). The pH was adjusted as needed with 0.1 N KOH or 0.1 N HCl. All solutions were aerated with individual aquarium air pumps (Elite 801, Rolf C. Hagen, Mansfield, Mass.) connected using tygon tubing located at the bottom of the 2-L

plastic bottles. The airflow was adjusted to $1 \text{ L} \cdot \text{min}^{-1}$. Solutions were changed weekly and the study was conducted for 16 weeks.

Physiological Measurements

Root FCR activity was monitored every 10 d as described by Chaney et al. (1972). White root tips $\sim 1 \text{ cm}$ long were collected from each plant, placed in a beaker filled with ice water for approximately 5 min, and transferred to the laboratory. To reduce the amount of time between excision and root FCR measurements, plants were sampled and brought to the laboratory in sets of 6 samples. The root tips were weighed and about 100 mg tissue fresh weight was placed in a test tube. The root tips were rinsed for 5 min with 2 ml of 0.2 mM CaSO_4 and placed in 2 ml of the assay solution containing 5 mM MES buffer (pH 5.5), 0.1 mM Fe(III)-EDTA , 10 mM CaSO_4 , and 0.3 mM sodium bathophenanthrolinedisulfonic acid (Na-BPDS). The samples were kept in the assay solution in the dark at 23°C in a shaker water bath for 1 h (50 rpm). The amount of Fe(II)-BPDS formed was measured colorimetrically at 535 nm. Values were standardized using a blank assay solution without roots. The concentration of Fe(II)-BPDS produced was calculated using the molar extinction coefficient of $22.14 \text{ mM} \cdot \text{cm}^{-1}$ (Chaney et al., 1972), and root fresh weights to calculate Fe reduction activity.

Leaf chlorophyll index was determined with a chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd, Japan). Six of the most recently fully expanded apical leaves (young leaves) and mature leaves (from below the 7th node), respectively, were used for leaf chlorophyll index determinations and expressed as SPAD readings.

Leaf Fe concentration was determined by separately sampling young and mature leaves at the end of the experiment. Six to seven leaves per plant in each treatment were

sampled. Leaf samples were gently rinsed with deionized water for about 1 min. Samples were oven-dried at 70°C for 48 h. The dry samples were ground through a 40-mesh screen in a Wiley mill. The samples were prepared and processed for analysis using a dry ash procedure described by Hanlon et al. (1994). Approximately 0.5 g of ground sample was transferred to a 10 ml beaker and placed in a muffle furnace at 500°C for 10 to 12 h. After the sample was cooled, a few drops of 1N HCl were added to the ash, after which samples were rinsed with 1 N HCl and transferred to a volumetric flask and brought to a volume of 50 ml. The samples were mixed thoroughly, and then filtered through Whatman Q8 filter paper. Subsamples were transferred to 20 ml polyethylene scintillation vials for Fe analysis using an inductively coupled argon plasma spectrometer (Spectro-CIROS CCD, FTCEA000, Germany) at the IFAS Analytical Research Laboratory, University of Florida, Gainesville, Fla.

Iron uptake was determined weekly. Each time the solution was changed, the concentration of Fe remaining in the solution was determined by collecting samples from each bottle and placing them into 20 ml polyethylene scintillation vials at the beginning and the end of the week. Samples were analyzed using an atomic absorption spectrophotometer (Perkin Elmer 3030B, Norwalk, Conn.) at the IFAS Analytical Research Laboratory, University of Florida, Gainesville, Fla. The depletion of Fe from the nutrient solution was calculated and represented Fe uptake. Values were adjusted for evaporation losses.

Growth Measurements

The total length of the main root and stem were measured at the beginning and the end of the experiment. Root length was measured basipetally from the media surface and stem length from the media surface to midpoint the apical bud. At the beginning of the

experiment, the fresh weight of the whole plant was determined. At the end of experiment, fresh and dry weights of young and mature leaves, stems (including axillary branches and shoots), and roots were determined using a Mettler BB240 electronic scale (Mettler, Greifensee, Switzerland). Tissue samples were oven-dried to a constant weight at 70°C.

Statistical Analysis

Treatments were arranged as a 2 (species) x 2 (Fe, presence or absence) factorial experiment with Fe and species as the main effects. The treatments were arranged in a completely randomized design with six single-plant replications for each treatment combination. Leaf chlorophyll index and root FCR activity were analyzed as a split split-plot design. Data were analyzed by analysis of variance, covariance, and multiple comparison tests using LSMEANS ($P < 0.01$) using SAS (SAS Institute, Cary, N.C.) statistical software. Regression analysis was done using SigmaPlot program (SPSS Science, Chicago, Ill.).

Results and Discussion

Physiological Measurements

There was a significant interaction between species and Fe concentration in the nutrient solution for almost all variables measured. Root FCR activity of pond apple grown without Fe for several days, which developed visible symptoms of chlorosis, decreased from 31 nmol of $\text{Fe}^{2+} \cdot \text{g}^{-1}$ of $\text{FW} \cdot \text{h}^{-1}$ on day 1 (prior to applying Fe treatments) to approximately 17 nmol of $\text{Fe}^{2+} \cdot \text{g}^{-1}$ of $\text{FW} \cdot \text{h}^{-1}$ and it was generally lower than root FCR activity in Fe-sufficient pond apple plants throughout the experiment (Fig. 6-1). Soursop plants grown with 90 μM Fe(III)-DTPA generally had higher root FCR activity than those of Fe-deficient plants. The root FCR activity of soursop was significantly higher

than that of pond apple in nutrient solution with Fe, having the highest activity on day 40, with a transient increase on day 80. These transient increases may be explained because plants can “turn on or off” physiological responses related to Fe uptake, such as root FCR activity, once sufficient levels of Fe have been obtained to maintain adequate Fe concentrations in plant tissue (Jolley et al., 1996). There were no significant differences in root FCR activities between species when grown in the Fe deficient nutrient media.

Soursop grown without Fe exhibited a decrease in root FCR activity within 10 d after treatments were initiated. In this respect, soursop and pond apple differ from many herbaceous plants (Römheld and Marschner, 1983; Romera et al., 1992; Zaharieva and Römheld, 2000) and other fruit crops (Manthey et al., 1994; Castle and Manthey, 1998; Pestana et al., 2001; Marler et al., 2002), which show an increase in root FCR activity under Fe deficient conditions. The increase of an inducible plasma membrane-bound FCR enzyme is an important part of the Strategy I plant responses to Fe-stress (Moog and Brüggemann, 1994; Marschner and Römheld, 1995). Under our conditions, root FCR activity decreased when plants were grown without Fe, indicating they were unable to respond to lack of Fe by increasing their Fe-reducing capacity. The results of this study agree with those on other tree species, such as peach rootstocks (Romera et al., 1991b; Gogorcena et al., 2000), pear, and quince (Tagliavini et al., 1995), where the lack of Fe does not always result in an increase in root FCR activity. There is considerable variability in root FCR activity among woody plant species in response to Fe deficiency. For *Ficus* growing with no Fe in the media, root FCR activity was 5,000 nmol Fe²⁺·g⁻¹ FW·h⁻¹ (Rosenfield et al., 1991). For some peach clones grown without Fe, root FCR activity was between 2,500 and 6,000 nmol Fe²⁺·g⁻¹ FW·h⁻¹ (De la

Guardia et al., 1995). In contrast, root FCR activity of pear growing without Fe was $29 \text{ nmol Fe}^{2+} \cdot \text{g}^{-1} \text{ FW} \cdot \text{h}^{-1}$ (Tagliavini et al., 1995) and quince growing without Fe was only $3 \text{ nmol Fe}^{2+} \cdot \text{g}^{-1} \text{ FW} \cdot \text{h}^{-1}$ (Tagliavini et al., 1995). *Ficus* and peach clones are considered Fe-deficiency-tolerant genotypes since FCR activity in the roots increased in response to lack of Fe compared to plants grown with Fe in the media (Rosenfield et al., 1991; De la Guardia et al., 1995). Pear and quince are Fe-deficiency-susceptible genotypes because their root FCR activities were not induced by a lack of Fe (Tagliavini et al., 1995).

Other studies have also found higher root FCR activity for plants treated with a low concentration of Fe compared with those grown without Fe. The addition of $2 \mu\text{M}$ Fe to plants grown without Fe produced large increases in root FCR activity of tomato (Zouari et al., 2001). Some studies have suggested that some Fe may be required for plants to develop responses to Fe deficiency with respect to the root FCR activity (Alcántara et al., 2000; Gogorcena et al., 2000). A complete lack of Fe may negatively affect ethylene activity because ethylene is somehow involved in activity of FCR transcription, or some of its components or regulatory elements (Romera et al., 1998, 1999). Moreover, Robinson et al. (1999) identified a flavocytochrome as one of the proteins responsible for FCR activity in *Arabidopsis*, which suggests the lack of Fe may negatively affect functioning of the FCR enzyme.

The differential abilities of soursop and pond apple to reduce Fe probably reflect differences in the properties of their standard reductase system and may provide an explanation for the different degrees of tolerance to Fe deficient soils observed between these species under field conditions. Although it has potential as a flood-tolerant rootstock for commercial *Annona* species, pond apple is not considered a commercial

crop, and its growing region is primarily restricted to its native wetland habitat. In these wetland areas, even in calcareous soils, there is sufficient soluble Fe in the soil for plant growth and development (Larson et al., 1991c). Therefore in its native habits there was no selection pressure for pond apple to evolve physiological mechanisms for Fe uptake and reduction that were as efficient as those in soursop, which is native to and cultivated in drier areas.

A relationship between Fe reductase and Fe uptake was not found in this study because Fe uptake was variable in both species (data not shown). The variability may have been due to the use of the depletion method to measure Fe uptake. Differences in uptake would have to be fairly large in order to be detected by measuring depletion from the nutrient solution.

Leaf chlorophyll index was similar in both species with and without Fe until the third week after treatments were initiated (Fig. 6-2). Leaf chlorophyll index of both pond apple and soursop leaves was significantly reduced by Fe stress. However, the most apical leaves of pond apple grown without Fe developed earlier and more severe chlorosis than soursop leaves grown without Fe. The severity of visual symptoms of pond apple Fe deficiency consistently increased until the end of the experiment, when the leaves were completely yellow (low leaf chlorophyll index). A highly significant linear relationship between extractable leaf chlorophyll and leaf chlorophyll index (SPAD readings) has been reported in *Annona* species (Schaper and Chacko, 1991). No significant differences were detected between leaf chlorophyll index of pond apple and soursop grown with Fe. The mature leaves of pond apple grown without Fe were dark green during the entire study (data not shown).

Sixteen weeks after treatments were initiated, leaf Fe concentration in soursop was significantly lower in plants grown without Fe (53 mg·kg⁻¹ dry wt) than those grown with Fe (71 mg·kg⁻¹ dry wt) (Fig. 6-3). Similarly, lack of Fe significantly reduced leaf Fe concentration of pond apple (23 mg·kg⁻¹ dry wt) compared with the Fe sufficient plants (49 mg·kg⁻¹ dry wt). There was no significant difference between leaf Fe concentration of soursop without Fe and pond apple with Fe. Values of leaf Fe concentration between 50 and 70 mg·kg⁻¹ have been reported as the sufficient range for other *Annona* species such as atemoya (*A. squamosa* L. x *A. cherimola* Mill) (Nakasone and Paull, 1998).

A positive linear correlation ($r^2 = 0.65$) existed between Fe concentration and leaf chlorophyll index (SPAD readings) in young leaves of pond apple and soursop (Fig. 6-4). Leaf chlorosis is reported to be inversely related to leaf Fe concentration in other crops as well (Tagliavini et al., 1995; Romera et al., 1991a; Alcántara et al., 2000).

Growth Measurements

Shoot length of Fe-deficient pond apple plants was significantly reduced compared with Fe-sufficient plants by the end of the experiment (Fig. 6-5). The negative effect of Fe deficiency on plant growth has also been reported in woody plants (De la Guardia et al., 1995; Alcántara et al., 2000), and herbaceous crops (Zouari et al., 2001). However, shoot length of soursop was not affected by lack of Fe. Soursop produced very little growth with or without Fe, and had a lower growth rate than pond apple with or without Fe. No differences were observed in root length within the species (data not shown), although new roots were produced continuously throughout the study.

Soursop and pond apple plants both grew over the experimental period as indicated by the increase in total plant fresh weights. However, pond apple grew considerably more

than soursop (Table 6-1). This different growth rate between soursop and pond apple has also been observed in plants grown in potting media in the glasshouse conditions. Also, the addition of Fe to the nutrient solution resulted in increased growth of pond apple, whereas, soursop was not affected by increased Fe in the nutrient media.

Root, stem, and leaf dry weights of pond apple were also lower for Fe-deficient plants than for Fe-sufficient plants (Table 6-2). However, root and stem dry weights of pond apple without Fe were significantly greater than those of soursop. There were no differences in organ dry weights between Fe and non-Fe treated soursop plants. Reduced dry weights and shoot length of pond apple grown without Fe can be explained by low leaf Fe concentration. A reduction in organ dry weight due to Fe deficiency has also been reported in other crops (Alcántara et al., 2000).

The differences in the intensity of responses between the two *Annona* species may partially explain the differences in tolerance to Fe-deficient soils. Pond apple plants grown with Fe exhibited lower leaf Fe concentrations than soursop under the same conditions suggesting that pond apple requires high Fe levels to maintain its rapid growth rate. Similarly, May et al. (1994) in strawberry reported that a decline in P, Mg, and Mo foliar concentrations during the rapid growth stage suggested an increase in demand for those particular nutrients. The limited effect of Fe deficiency on shoot length, fresh and dry weights of soursop may explain why soursop did not exhibit severe chlorosis. Soursop plants grown under Fe stress could maintain a more favorable nutrient concentration due to a slower growth rate, which could be advantageous in Fe-deficient soils. Therefore, the absolute values for nutritional threshold levels for each species may also depend on the nutrient demand to sustain plant growth. Alternatively, there may

have been a sufficient amount of stored Fe in soursop plants prior to treatment initiation, since the plants were 4 months-old.

Conclusions

Root FCR activity of pond apple and soursop was not induced by lack of Fe. Thus, they appear to be Fe-deficiency susceptible species based on the result from this study. Pond apple grown with or without Fe had relatively low root FCR activity probably due to its native wetland habitat where there is sufficient soluble Fe in the soil for plant growth and development (Larson et al., 1991c). Therefore, pond apple did not need to develop physiological mechanisms for Fe reduction that were as efficient as those in soursop grown with Fe, which is native to and cultivated in drier areas. Lack of Fe in the nutrient solution resulted in a lower leaf chlorophyll index, Fe concentration, and growth of pond apple. The lack of Fe did not affect these factors for soursop.

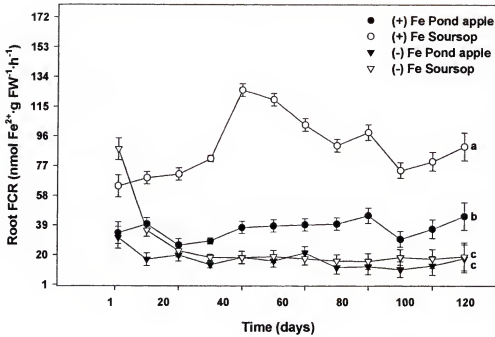


Figure 6-1. Effect of Fe concentration in the nutrient solution on root Fe chelate reductase (FCR) activity of *Annona* species over time. Data are means \pm SE, $n=6$ plants. For each species, different letters indicate significant differences among means by LSMEANS, ($P \leq 0.01$) on the last measurement date (week 16).

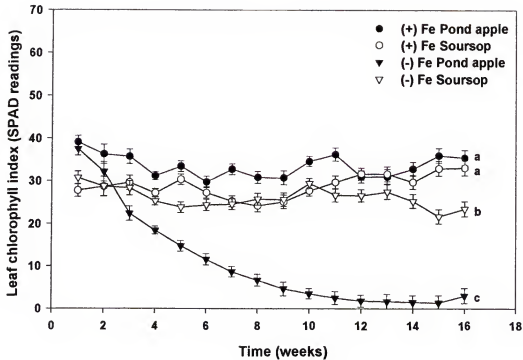


Figure 6-2. Effect of Fe concentration in the nutrient solution on leaf chlorophyll index (SPAD readings) in young leaves of *Annona* species over time. Data are means \pm SE, $n=6$ plants. For each species, different letters indicate significant differences among means by LSMEANS, ($P \leq 0.01$) on the last measurement date (week 16).

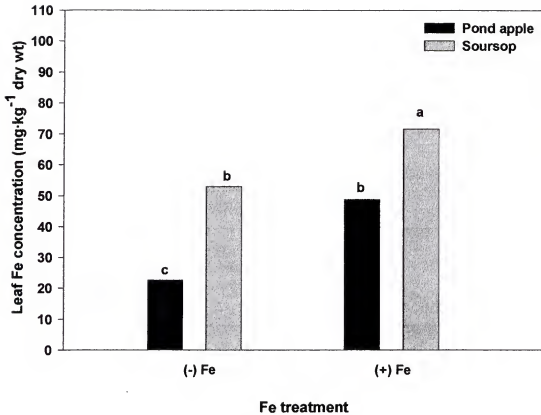


Figure 6-3. Effect of Fe concentration in the nutrient solution on leaf Fe concentration in young leaves of *Annona* species 16 weeks after Fe treatments were initiated. For each species, different letters above the bars indicate significant differences among means by LSMEANS ($P \leq 0.01$), $n=6$ plants.

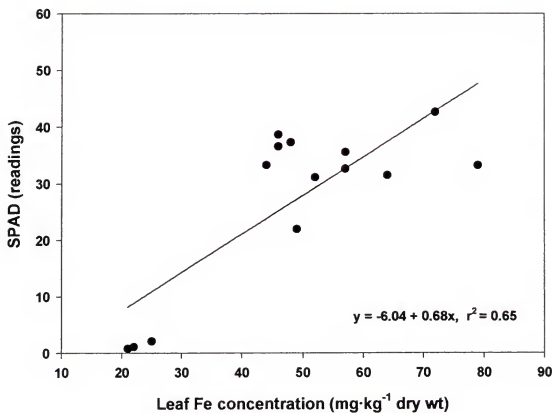


Figure 6-4. Relationship between leaf Fe concentration and SPAD readings in young leaves of *Annona* species 16 weeks after Fe treatments were initiated.

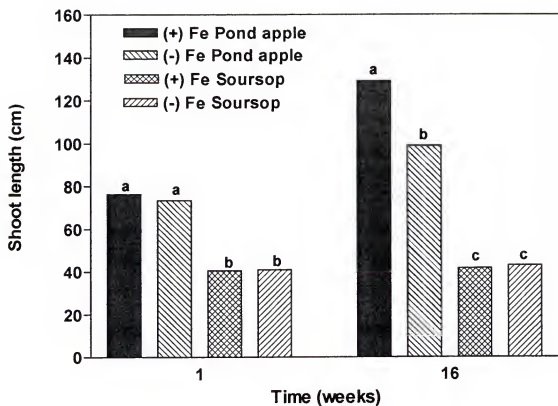


Figure 6-5. Effect of Fe concentration in the nutrient solution on shoot length of *Annona* species 16 weeks after Fe treatments were initiated. For each species, different letters above the bars indicate significant differences among means according to LSMEANS ($P \leq 0.01$), n=6 plants.

Table 6-1. Effect of Fe concentration in the nutrient solution on total plant fresh weights of pond apple and soursop at weeks 1 and 16.

Species	Treatments	Total plant fresh wt (g) ^z	
		Week 1	Week 16
Pond apple	Fe (-)	45.77a	176.16b
	Fe (+)	42.80a	314.22a
Soursop	Fe (-)	22.39a	37.73a
	Fe (+)	21.12a	36.75a

^zMeans within columns followed by different letters indicate significant differences by LSMEANS, ($P \leq 0.01$), n= 6 plants

Table 6-2. Effect of Fe concentration in the nutrient solution on dry weights of pond apple and soursop 16 weeks after treatments were initiated.

Species	Treatments	Dry wt (g) ^z		
		Root	Stem	Leaves
Pond apple	(-) Fe	14.90b	16.06b	3.67b
	(+) Fe	24.10a	24.94a	12.23a
Soursop	(-) Fe	4.67c	3.63c	2.41b
	(+) Fe	3.48c	3.69c	2.57b

^zMeans within columns followed by different letters indicate significant differences by LSMEANS, $P \leq 0.01$, n=6 plants.

CHAPTER 7

ROOT AND LEAF FERRIC CHELATE REDUCTASE ACTIVITIES OF TWO *Annona* SPECIES ARE REDUCED BY IRON DEFICIENCY

Introduction

The *Annonaceae* includes several subtropical and tropical fruit crop species with economic importance throughout the world. Two *Annona* species, pond apple (*A. glabra* L.) and soursop (*A. muricata* L.) have potential as flood-tolerant rootstocks for commercial annona crops (Núñez-Elisea et al., 1999).

In areas prone to cyclical flooding, such as southern Florida, *Annona* species are grown in calcareous soils, which limit the supply of soluble Fe (Lucena, 2000). Iron deficiency induces leaf chlorosis, and suppresses growth and yield of several crop species (Korcak, 1987; Tagliavini and Rombolá, 2001). The application of Fe chelates is often not the best method for preventing or ameliorating Fe chlorosis because of the high cost of chelates. Alternatively, development of techniques for screening tolerance to Fe deficiency and selecting plants that are tolerant of Fe deficiency may be an economical method to solve the problem (Jolley et al., 1996; Castle and Manthey, 1998).

Iron uptake is preceded by reduction of Fe^{3+} to Fe^{2+} before the reduced form can cross the plasmalemma of the outer root cells (Chaney et al., 1972). A second Fe reduction occurs before it enters the leaf cells (Brüggemann et al., 1993). This is a second important step before Fe is available for leaf metabolism. Higher plants use two different strategies to alleviate Fe deficiency, Strategy I (dicots and monocots, except the graminaceae) and Strategy II (graminaceae). Strategy I plants exhibit an increased

inducible root ferric chelate reductase (FCR) and proton pump activities, along with the release of reductants into rhizosphere induced by Fe stress (Marschner and Römheld, 1995). Strategy II plants (graminaceae species) are generally characterized by increases in the biosynthesis and secretion of compounds called phytosiderophores, which are highly effective as Fe(III) chelators. Recently, De Nisi and Zocchi (2000) have suggested a role of phosphoenolpyruvate carboxylase (PEPC) as another physiological response to Fe deficiency in calcareous soils. This enzyme incorporates bicarbonate into phosphoenolpyruvate, generating oxalacetate, which is a precursor of citrate and Fe is transported to the shoot as Fe^{3+} -citrate (Tiffin, 1970).

Most of the studies for screening Strategy I plants to Fe-deficiency tolerance have been carried out by treating plants in nutrient solutions without Fe to induce root FCR activity (Moog and Brüggeman, 1994). In our previous experiment (see Chapter 6), root FCR activity of pond apple and soursop was not induced by lack of Fe in the nutrient medium. Some studies have reported that low Fe concentration is needed to induce root FCR activity instead of the absence of Fe (Gogorcena et al., 2000; Zouari et al., 2001). In the our previous experiment (see Chapter 6), with 90 μM Fe in the nutrient solution the leaf Fe concentration of pond apple and soursop were still in the sufficiency range as reported for other *Annona* species (George et al., 1987). Thus, in this study, to determine Fe deficiency tolerance of pond apple and soursop, 45 μM Fe was selected as the maximum concentration to evaluate how much the Fe in the nutrient solution could be reduced while still maintaining adequate leaf Fe concentration. Additionally, evaluating leaf FCR activity may provide a better understanding of the ability of these *Annona* species in reducing Fe and its subsequent availability for leaf metabolism.

Information is lacking concerning tolerance of *Annona* species to low Fe concentrations, which is a widespread problem in calcareous soils throughout the world. The objective of this study was to compare the effect of Fe nutrition on root and leaf FCR activities in pond apple and soursop, two *Annona* species from different habitats.

Materials and Methods

The study was conducted from April to July 2002 in a sunlit glasshouse in Gainesville, Fla. Air temperature and relative humidity were monitored using a Hobo H8 Pro Series (Onset Computer Corporation, Pocasset, Mass.) temperature logger. Average day/night air temperatures during the experimental period ranged from 32/25 to 22/20°C, and relative humidity was 80 to 90%.

Plant Material

One-year-old seedlings of pond apple (*A. glabra* L.) and soursop (*A. muricata* L.) were used. Prior to applying treatments, plants were fertigated monthly with 3.7 g·L⁻¹ of water-soluble fertilizer (14.5N-8P-16.6K, with ammonium, nitrate, and urea as N sources).

Treatments

Fifteen uniform trees of each species were selected, and their root systems washed. Prior to initiating treatments, plants were acclimated in a complete nutrient solution containing 2 µM Fe for 3 weeks in 3-L plastic bottles. The plastic bottles were wrapped with aluminum foil to prevent algal growth. After acclimation in the nutrient solution, roots were soaked in 1 mM NaEDTA for 5 min to remove apoplastic Fe and washed three times in DI water (Rosenfield et al., 1991) before treating them with a complete nutrient solution containing 2 (low), 22.5 (medium) or 45 (high) µM Fe(III)-DTPA as the Fe source. The nutrient solution also contained 0.5 M NaNO₃, 0.05 M K₂HPO₄·3H₂O, 0.1 M

MgSO₄·7H₂O, 0.05M CaCl₂·2H₂O, 0.0045 M H₃BO₃, 0.001 M MnSO₄·H₂O, 0.001 M ZnSO₄·7H₂O, 0.0003 M CuSO₄·5H₂O, 0.2 μM Na₂MoO₄·2H₂O. The solution pH was buffered with 7 mM MES at pH 6.5. The pH of 6.5 was monitored daily with a standard electrode attached to a pH meter (Accumet AP62, Fisher Scientific, Pittsburgh, Pa.) and maintained using 0.1 N KOH or 0.1 N HCl. All solutions were aerated with individual aquarium air pumps (Elite 801, Rolf C. Hagen, Mansfield, Mass.) connected using tygon tubing located at the bottom of the 3-L plastic bottles. The airflow was adjusted to 1 L·min⁻¹. Solutions were changed weekly and the treatment period was 12 weeks.

Physiological Measurements

Root FCR activity was monitored every 10 d as described by Chaney et al. (1972). White root tips, about 1 cm long, were collected from each plant, placed in a beaker filled with ice water for approximately 5 min, and transferred to the laboratory. To reduce the amount of time between excision and root FCR measurements, plants were sampled and brought to the laboratory in sets of 6 samples. The roots were weighed and about 100 mg of fresh tissue was placed in a test tube. The root tips were rinsed for 5 min with 2 ml of 0.2 mM CaSO₄ and placed in 2 ml of the assay solution containing 5 mM MES buffer (pH 5.5), 0.1 mM Fe(III)-EDTA, 10 mM CaSO₄, and 0.3 mM sodium bathophenanthrolinedisulfonic acid (Na-BPDS). The samples were kept in the assay solution at 23°C in a shaker bath for 1 h (50 rpm) in darkness. After the incubation period, a 1 ml aliquot from each tube was transferred to a cuvette and absorbance was determined spectrophotometrically at 535 nm (Shimadzu UV-160, Japan). Values were standardized using a blank assay solution without roots. The concentration of

Fe(II)-BPDS produced was calculated using the molar extinction coefficient of 22.14 mMcm^{-1} (Chaney et al., 1972), and root fresh weights to determine Fe reduction activity.

For leaf FCR activity, five mm diameter discs from fully expanded leaves from the apical position of each plant were obtained using a cork borer. Leaf discs were placed in a beaker filled with ice water and transported to the laboratory where they were weighed and approximately 100 mg fresh weight tissue was placed in test tubes containing 0.2 mM CaSO_4 . The CaSO_4 was discarded after 10 min, and 2 ml of assay solution as described for root FCR activity were added to the tubes. Samples were vacuum infiltrated for 10 min in the light before incubating in a shaker bath at 50 rpm and 23°C for 1 h in darkness. After incubation, a 1 ml aliquot from each tube was transferred to a cuvette and absorbance was determined spectrophotometrically at 535 nm. The concentration of Fe(II)-BPDS was calculated using the same method as described for root FCR activity.

Leaf chlorophyll index was determined with a chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd, Japan). Six recently fully expanded apical leaves (young leaves) and six mature leaves (from below the 7th node), respectively, were used to measure leaf chlorophyll index.

Leaf Fe concentration was determined by separately sampling six to seven recently fully expanded apical leaves (young leaves) and six mature leaves (from below the 7th node) at the end of the experiment. Six to seven leaves per plant in each treatment were sampled. Leaf samples were gently rinsed with deionized water for about 1 min. Samples were oven-dried at 70°C for 48 h. The dry samples were ground through a 40-mesh screen in a Wiley mill. The samples were prepared and processed for analysis using a dry ash procedure (Hanlon et al., 1994). Approximately 0.5 g of ground sample was

transferred to a 10 ml beaker and placed in a muffle furnace at 500°C for 10 to 12 h. After the sample was cooled, a few drops of 1N HCl were added to the ash, after which samples were rinsed with 1 N HCl and transferred to a volumetric flask and brought to a volume of 50 ml. The samples were mixed thoroughly, and then filtered through Whatman Q8 filter paper. Subsamples were transferred into 20 ml polyethylene scintillation vials for Fe analysis using an inductively coupled argon plasma spectrometer (Spectro-CIROS CCD, FTCEA000, Germany) at the IFAS Analytical Research Laboratory, University of Florida, Gainesville, Fla.

Growth Measurements

Plant height (total length of the main stem) was measured basipetally from the apical bud at the beginning and the end of the experiment.

Statistical Analysis

Treatments were arranged as a 2 (species) x 3 (Fe concentrations) factorial with Fe and species as the main effects. The treatments were arranged in a completely randomized design with five single-plant replications for each treatment combination. Root and leaf FCR activities and leaf chlorophyll index (SPAD readings) were analyzed as split split-plot design. Data were analyzed by a combination of statistical tests, including analysis of variance, multiple comparisons using LSMEANS ($P \leq 0.05$), and covariance procedures using SAS (SAS Institute, Cary, N.C.) statistical software. Regression analysis was also done using the SigmaPlot program (SPSS Science, Chicago, Ill.).

Results and Discussion

Physiological Measurements

Pond apple grown with low Fe (2 μ M) in the nutrient solution developed symptoms of leaf chlorosis, and its root FCR activity decreased drastically from 44.77 nmol Fe²⁺·g⁻¹ FW·h⁻¹ on day 1 (prior to applying Fe treatments) to 9.57 nmol Fe²⁺·g⁻¹ FW·h⁻¹ on day 80 (Fig. 7-1A). In general, root FCR activity in pond apple followed a similar pattern over time for all Fe concentrations in the nutrient solution, except at day 40 when plants grown with 45 μ M Fe in the nutrient solution showed significantly higher root FCR activity than plants in the other treatments. The decline in root FCR activity of pond apple was greatest between days 60 and 80 regardless of Fe concentrations. Similarly, root FCR activity of soursop grown with a low concentration Fe (2 μ M) declined from 27.23 nmol Fe²⁺·g⁻¹ FW·h⁻¹ on day 1 to 14.35 nmol Fe²⁺·g⁻¹ FW·h⁻¹ on day 80 (Fig. 7-1B). Patterns of root FCR activity in soursop were similar for all Fe treatments over time.

Increased root FCR and proton pump activities, along with the release of reductants into the rhizosphere resulting from Fe deficiency have long been considered the main responses induced by Strategy I plants to Fe deficiency (Marschner and Römheld, 1995). In this study, root FCR activity of both species was not induced by Fe deficiency (2 μ M Fe). However, this response may not be sufficient to classify these *Annona* species as non-Fe-deficiency tolerant. Recent findings suggest using caution when evaluating root FCR activity as a screening technique for selecting rootstocks for Fe tolerance (Alcántara et al., 2000) because root FCR activity is not always related to leaf Fe-deficiency tolerance. Additionally, the results of this study are consistent with those for

other woody plants such as some peach rootstocks (Romera et al., 1991b; Gogorcena et al., 2000), pear and quince (Tagliavini et al., 1995), where root FCR activity was not always induced or as high as that found in some annual plants under Fe deficiency. The low FCR activity in some woody plants may be because trees store large amounts of Fe in their roots (Mengel, 1994); thus plants may not need to activate physiological mechanisms to obtain Fe. Some studies have shown that root FCR activity is more likely to be induced at very low Fe concentrations than by the absence of Fe in the nutrient solution (Zouari et al., 2001), and that Fe concentration depends on the species (Chaney et al., 1972; Romera et al., 1996; Zouari et al., 2001; Gogorcena et al., 2000; Bohórquez et al., 2001). The highest root FCR activity for pond apple was induced at 45 μM Fe on day 40, suggesting that pond apple may need a high concentration of Fe to induce root FCR activity. However, the high root FCR activity at 45 μM Fe was transient because root FCR activity has been positively related to Fe uptake (Manthey et al., 1994) and Fe uptake rate can be adjusted when the plant's demand is satisfied (Schmidt and Steinbach, 2000).

In some peach rootstocks there were no consistent relationships between root FCR activity of the whole root plant and that of the root tips because some root tips may be reducing much more Fe than others (Gogorcena et al., 2000). Thus, for some species it may be necessary to use the whole root system for determining FCR activity. This integrates information from many root tips at the same time. This may explain the high variation (high SE) of root FCR activity in soursop among Fe treatments observed in this study (Fig. 7-1B).

A more rapid decline in root FCR activity of pond apple than in soursop by Fe deficiency probably reflects differences in the properties of their Fe reductase systems and may explain the different degrees of tolerance to Fe deficient soils observed between these species under field conditions. Pond apple exhibits severe leaf chlorosis when it is grown in non-flooded calcareous soils (B. Schaffer, personal communication).

The overall mean leaf FCR activity of pond apple was significantly less than that of soursop throughout the study ($P \leq 0.05$). For each species leaf FCR activity was not affected by Fe concentration in the nutrient solution until the end of the study (80 d), when plants grown at the low Fe concentration ($2\mu\text{M}$) had lower leaf FCR activity than plants grown at the other Fe concentrations (Fig. 7-2A and B). The results confirm the existence of FCR activity in leaf discs of pond apple and soursop as a prerequisite for Fe^{3+} reduction to Fe^{2+} before the reduced form can cross the plasmalemma into the leaf cell. Leaf FCR activity has also been reported in other crops, including cowpea (Brüggemann et al., 1993), sunflower (De la Guardia and Alcántara, 1996), sugar beet (Larbi et al., 2001; González-Vallejo et al., 2000), and kiwifruit (Rombolá et al., 2000).

Results in pond apple and soursop were similar to those found in sunflower (De la Guardia and Alcántara, 1996), and kiwifruit (Rombolá et al., 2000), where FCR activity in leaf discs was lower in Fe-deficient plants than in Fe-sufficient plants. The lower leaf FCR activity in Fe-deficient plants than in Fe-sufficient plants may be in part due to fewer reducing agents in the leaf cytosol cell of Fe-deficient plants. It is possible that the same electron donors in root cells such as NADH and NADPH (Moog and Brüggemann, 1994; Manthey, 1992) are also involved in leaf Fe reduction reactions. Chloroplast and mitochondria are the main sources for leaf cytosolic reductant of NAD(P)H for the leaf

reduction of other elements such as NO_3^- (Huppe and Turpin, 1994). For instance, it has been reported that chloroplasts reduce Fe from Fe(III) chelates (Bughio et al., 1997). Therefore, Fe deficiency may have reduced the production and exportation of reducing agents to the cytosol, since almost all of a plant's Fe is in the chloroplast (Terry and Abadía, 1986), mainly in the thylakoid membranes (Terry and Low, 1982).

Higher leaf FCR activity in soursop than in pond apple suggests that soursop is more efficient than pond apple in reducing Fe^{3+} -citrate to Fe^{2+} before it is transported across plasmalemma into leaf cytosol cells, resulting in more Fe availability for leaf metabolism. This is important since Fe availability in leaves is not uniform over time because its translocation is dependent on the transpiration stream, which varies during the day. Leaf Fe can be utilized for chlorophyll synthesis and maintaining chloroplast structure and function (Abadía, 1992), which also affect net CO_2 assimilation (Terry, 1980; Spiller and Terry, 1980).

It is not clear whether FCR expressed in leaf cells is identical with that in the root epidermis (Schmidt, 1999). These results with pond apple and soursop suggest that their leaf Fe uptake mechanisms have some characteristics different from those of roots, since soursop showed higher leaf FCR activity than pond apple which was not observed for root FCR activity.

There was a significant interaction between Fe concentration and species for leaf chlorophyll index in the youngest apical leaves. However, mature leaves were not affected by Fe treatments (data not shown). This response may be a result of Fe immobility resulting in chlorosis in young leaves. Leaf chlorophyll content of pond apple decreased much more rapidly over time at a low Fe concentration ($2 \mu\text{M Fe}$) in the

nutrient solution than at the other Fe concentrations (Fig. 7-3A). The decline began 6 weeks after Fe treatments were initiated, presumably because 1-year-old pond apple seedlings could have some stored Fe. The severity of visual symptoms of Fe deficiency increased until the end of the study when leaves became completely yellow (low leaf chlorophyll index). In contrast, the leaf chlorophyll index of soursop was not affected by Fe concentration in the nutrient solution throughout the study (Fig. 7-3B).

Based on SPAD values, it can be concluded that low Fe concentration in the nutrient solution decreased leaf chlorophyll content because a highly significant linear relationship between extractable leaf chlorophyll and leaf SPAD readings has been reported in other *Annona* species (Shaper and Chacko, 1991). Leaf chlorosis in pond apple grown at a low Fe concentration in the nutrient solution appears associated with the low root and leaf FCR activities found in plants grown at low Fe concentrations (Fig 7-1A, 7-2A). The growing leaves invest a high proportion of Fe in the chloroplast membranes and less Fe in stromal storage compared with mature leaves (Terry and Abadía, 1986). Thus, as Fe deficiency develops, chlorophyll declines as consequence of the decreased thylakoids.

There were highly significant effects of Fe concentration in the nutrient solution on leaf Fe concentration (Fig. 7-4). Leaf Fe concentration in soursop was significantly lower in plants grown at 2 μM (35 $\text{mg}\cdot\text{kg}^{-1}$ dry wt) than in plants grown at 22.5 or 45 μM (57 and 57.40 $\text{mg}\cdot\text{kg}^{-1}$ dry wt), respectively. Although leaf Fe concentration was reduced at low Fe concentration, soursop did not exhibit leaf chlorosis symptoms (Fig 7-3B). Low Fe concentration in the nutrient solution significantly reduced leaf Fe concentration in pond apple (30 $\text{mg}\cdot\text{kg}^{-1}$ dry wt), which developed chlorosis symptoms. Leaf Fe

concentrations of pond apple and soursop at 2 μM Fe were lower than those reported as sufficiency range for other *Annona* species such as atemoya (*A. squamosa* L. x *A. cherimola* Mill.) (George et al., 1987).

Soursop tended to have a higher leaf Fe concentration than pond apple, which may be due to the higher leaf FCR activity observed in soursop than in pond apple leaves. In calcareous soils, pond apple requires application of more chelated Fe to the soil than commercial *Annona* species such as atemoya (B. Schaffer, personal communication). The high Fe requirement of pond apple may be associated with its native wetland origin (Morton, 1987; Nakasone and Paull, 1998). In saturated calcareous soils Fe^{3+} is reduced to Fe^{2+} and it is more soluble Fe form (Larson et al., 1991c). Thus, pond apple probably evolved without Fe limitations on plant growth and development.

Leaf Fe concentration of mature pond apple and soursop leaves showed similar pattern as young leaves (data not shown).

Growth Measurements

Height of pond apple plants was less for plants grown with 2 μM Fe than for plants grown with 22.5 or 45 μM Fe in the nutrient solution (Fig. 7-5). Reduced plant growth due to Fe deficiency has also been reported for other fruit crops such as peach (De la Guardia et al., 1995; Alcántara et al., 2000; De la Guardia and Alcántara, 2002) and olive (De la Guardia and Alcántara, 2002) because a low Fe supply reduces the chlorophyll concentration, thereby decreasing photosynthesis and potential growth. Iron is an essential cofactor for numerous enzymes, and therefore various biochemical reactions may be decreased by an insufficient Fe supply. Iron is also a constituent of ribonucleotide reductases (RNR) (Nordlund et al., 1990; Reichard, 1993), which produce the

deoxyribonucleotides needed for DNA synthesis. Therefore, if the synthesis of this enzyme is restricted due to a lack of Fe, meristematic growth (cell division) is also restricted because DNA synthesis is reduced. Reactions such as DNA synthesis and chlorophyll synthesis may compete for Fe. Thus, the reduced growth of pond apple at low Fe concentration may be attributed to its effect on DNA production of new cells and decreased leaf chlorophyll content.

Height of soursop plants was not affected by the Fe concentration in the nutrient solution, suggesting that soursop may tolerate Fe-deficient conditions. Soursop had a slower growth rate than pond apple, which has also been observed in plants grown in potting media in the glasshouse conditions. The ability of soursop to tolerate Fe deficient conditions and to grow slower than pond apple may be advantageous when using soursop as a rootstock (Núñez-Elisea et al., 1999), since vigorous rootstocks may induce excessive growth in the scion and reduce yields.

The different responses between the two *Annonas* species may partially explain the different tolerance to Fe-deficient soils. Pond apple had lower leaf Fe concentrations than those reported as the sufficiency range for other *Annona* species such as atemoya growing under the same conditions as soursop. This suggests that pond apple requires high levels of Fe to maintain its rapid growth rate. The slow growth rate and high leaf FCR activity of soursop may presumably enable this species to tolerate Fe deficiency and thus not exhibit as severe leaf chlorosis as pond apple.

Conclusions

Root and leaf FCR activities of pond apple and soursop are reduced by Fe deficiency. Pond apple had relatively low root and leaf FCR activities probably due to its native wetland origin, where there is sufficient soluble Fe for plant growth and

development. A higher leaf FCR activity in soursop than in pond apple suggests that soursop is more efficient in reducing Fe, resulting in more availability of Fe to be used for leaf metabolism. In this study, pond apple developed rapid leaf chlorosis compared to soursop indicating that pond apple is an Fe-deficiency intolerant species. Although soursop did not exhibit leaf chlorosis, the results from this study may not be sufficiently conclusive to classify soursop as an Fe-deficiency tolerant species because stored Fe in 1-year-old plants may mask responses to Fe.

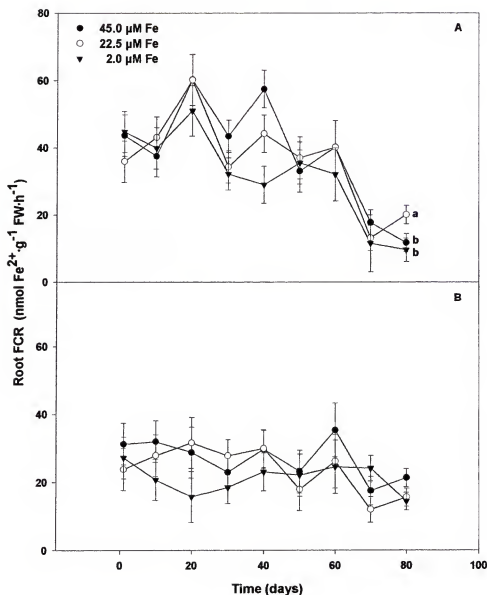


Figure 7-1. Effect of Fe concentration in the nutrient solution on root Fe chelate reductase activity (FCR) of pond apple (A), and soursop (B) over time. Symbols and error bars represent means \pm SE, $n=5$ plants. For each species, different letters indicate significant differences among means by LSMEANS, ($P \leq 0.01$) on the last measurement date (80 d). The absence of letters indicates no significant differences among means.

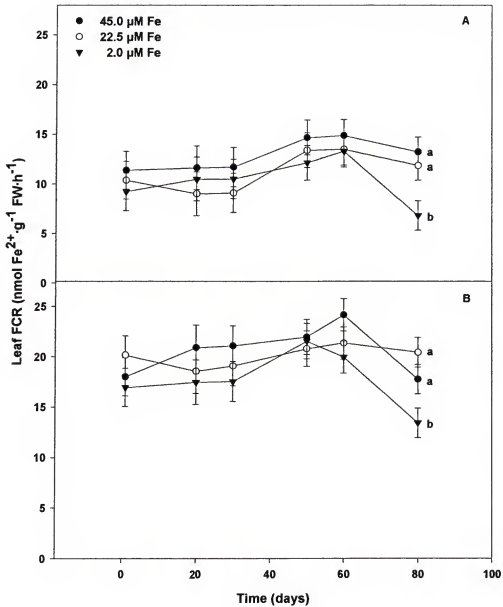


Figure 7-2. Effect of Fe concentration in the nutrient solution on leaf Fe chelate reductase activity (FCR) of pond apple (A), and sour sop (B) over time. Symbols and error bars represent means \pm SE, $n=5$ plants. For each species, different letters indicate significant differences among means by LSMEANS, ($P \leq 0.01$) on the last measurement date (80 d).

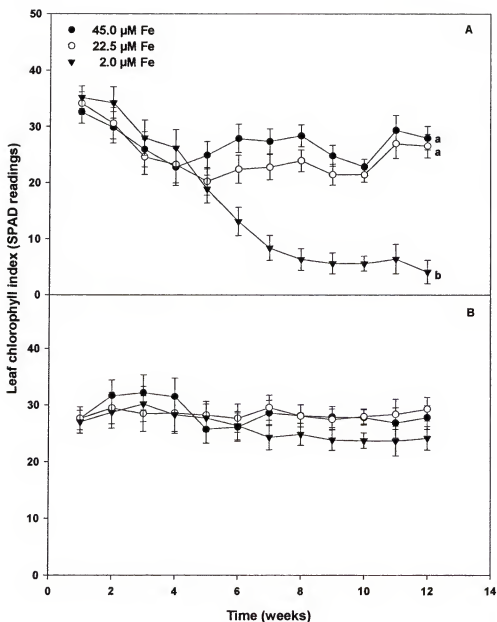


Figure 7-3. Effect of Fe concentration in the nutrient solution on leaf chlorophyll index in young leaves of pond apple (A), and soursop (B) over time. Symbols and error bars represent means \pm SE of 5 plants with 6 leaf samples per plant. For each species, different letters indicate significant differences among means by LSMEANS, ($P \leq 0.01$) on the last measurement date (12 weeks). The absence of letters indicates no significant differences among means.

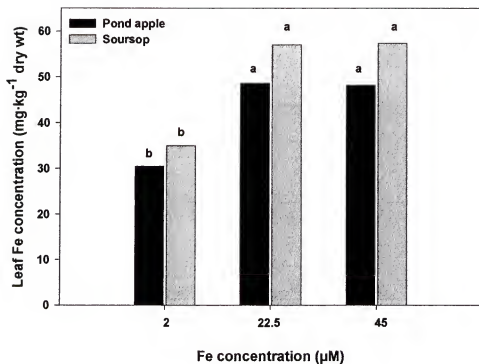


Figure 7-4. Effect of Fe concentration in the nutrient solution on leaf Fe concentration in young leaves of pond apple and soursop 12 weeks after Fe treatments were initiated. For each species, different letters above the bars indicate significant differences among means by LSMEANS ($P \leq 0.05$), $n=5$ plants.

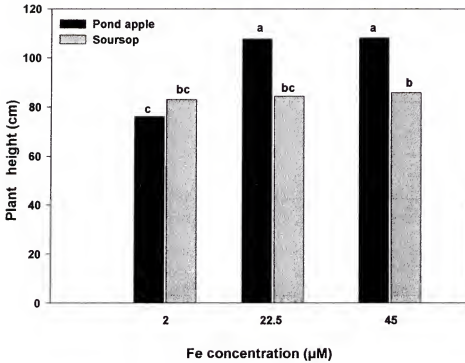


Figure 7-5. Effect of Fe concentration in the nutrient solution on height of pond apple and soursop 12 weeks after Fe treatments were initiated. For each species, different letters above the bars indicate significant differences among means by LSMEANS ($P \leq 0.05$), $n=5$ plants.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Pond apple (*Annona glabra* L.) and soursop (*A. muricata* L.) are two *Annona* species with potential as flood-tolerant rootstocks for commercial *Annona* species. Lack of tolerance of pond apple to Fe deficiency and susceptibility of soursop to low temperature ($<10^{\circ}\text{C}$) may restrict the use of these *Annona* species as flood-tolerant rootstocks. Iron deficiency and occurrence of temperatures below than 10°C during winter occur in agricultural soils around the world.

This study was conducted to evaluate the effects of soil temperature, flooding, and Fe deficiency stress on the physiology and growth of pond apple and soursop. This study would help assess the potential of these flood-tolerant rootstocks under a wide range of conditions and to possibly overcome any horticultural impediments to their use. Leaf chlorophyll index, net CO_2 assimilation, number of leaves, plant height, trunk diameter, and fresh and dry weights of containerized pond apple, soursop, and ‘Gefner’ atemoya on sugar apple rootstock trees were lowest at low soil temperatures (5 and 10°C) compared with higher temperatures (20 to 35°C). At soil temperatures of 5 and 10°C the amount of CO_2 lost by leaves during respiration was greater than the amount assimilated within 1 week after soil temperature treatments were initiated, resulting in reduced growth compared to plants grown at the higher soil temperatures. At low soil temperatures the leaf chlorophyll index of all species was decreased within 3 weeks of treatment initiation. Leaf chlorophyll index was associated with decreased net CO_2 assimilation responses of all species at the low temperatures.

The temperature responses observed in this study are consistent with the putative evolutionary center of origin of *Annona* species tested. Those of tropical origin such as soursop and atemoya on sugar apple rootstock had the greatest leaf chlorophyll index, net CO₂ assimilation, and growth at the highest soil temperatures (25 to 35°C). Pond apple, the only species tested indigenous to the subtropics, exhibited the highest leaf chlorophyll index, net CO₂ assimilation, and growth at soil temperatures of 20 to 25°C. Physiological and growth responses of 'Gefner' atemoya were strongly influenced by its sugar apple rootstock, which originated in the tropics.

A second study was conducted to evaluate the interaction of flooding and soil temperature on physiology and growth of pond apple and soursop. More rapid soil reduction was observed at soil temperatures of 10, 20, 25, and 35°C than at the 5°C. Three days after flooding treatments were initiated, soil Eh was below 200 mV at all soil temperatures, suggesting limited availability of O₂ in the root zone. Pond apple developed morphological adaptations to flooding, including hypertrophied trunk lenticels (especially at 25°C), upward root growth into the floodwater, and basal trunk swelling. Soursop also developed hypertrophied trunk lenticels (especially at 35°C), adventitious roots, and promotion of some basal shoots. These morphological adaptations may help the plants tolerate flooding conditions under optimal temperatures since plants of both species flooded and exposed to soil temperatures of 5 and 10°C did not show visible morphological adaptations, and died after 4 week of flooding. Although soursop could tolerate flooding at the higher temperatures, as evidenced by 100 % plant survival at 20, 25 and 35°C this species cannot be considered very flood tolerant since flooding resulted in increased root electrolyte leakage, and decreased leaf chlorophyll index, net CO₂

assimilation, and plant height. However, the development of certain morphological adaptations to flooding may help soursop tolerate and survive transitory flooding. In contrast, flooding had no effect on the leaf chlorophyll index, net CO₂ assimilation, and growth of pond apple. Flooded pond apple plants had a higher leaf chlorophyll index, net CO₂ assimilation, and growth at soil temperatures of 20 to 25°C than at 5, 10 or 35°C. Soursop had the least inhibited growth at 25 to 35°C soil temperatures.

Non-flooded pond apple plants in the second study tended to have higher growth rates at soil temperatures between 25 to 35°C compared to the first study when the greatest growth was observed mainly between 20 to 25°C. Plants used in the two studies came from different nurseries and pond apple is propagated sexually. Differences in plant growth and graft compatibility have been observed among different seed sources for pond apple (J.H. Crane, personal communication). Thus, highly variable responses among plants may be expected within this species.

Iron deficiency is a common problem in alkaline soils and the most effective and common method to correct this is by soil drench applications of ferric chelated Fe sources (i. e., Sequestrene-138, Fe-EDDHA). However, when these soils are flooded, significant chemical transformation occur resulting in increased solubility of Fe in the soil solution. Although prolonged flooding can reduce availability of some nutrients and consequently reduce plant growth, short-term flooding of limestone soils can result in increased uptake of some elements including Fe and Mn.

The effect of Fe source (chelated and non-chelated), Fe rate, and flooding on physiology and growth of pond apple was evaluated. Flooding for 12 weeks decreased leaf concentration of N, P, K, Ca, Mg, Zn, and Cu, and increased Fe and Mn in pond

apple plants. In general, plants treated with chelated Fe had a greater leaf chlorophyll index and growth than plants fertilized with non-chelated Fe. Growth of pond apple plants in all Fe and flooding treatments followed the same leaf chlorophyll index patterns, and leaf Fe concentration, except plants in the chelated, flooded treatments. Although, plants in the chelated, flooded treatments had high leaf Fe concentrations compared to the other Fe and flooding treatments, the leaf chlorophyll index and growth were not as great as expected. Although plants in the chelated, flooded treatment potentially had increased Fe^{2+} availability, Fe may be accumulated in the leaf apoplast since pond apple has low leaf ferric chelate reductase (FCR) activity. Low leaf FCR decreases Fe^{3+} reduction by mesophyll cells resulting in low Fe availability for the leaf metabolism. Pond apple plants in the chelated, non-flooded treatments had the greatest leaf chlorophyll index and growth compared to the other Fe and flooding treatments, with the optimum Fe rate for both variables at 2.5 to 5 g/plant. For flooded pond apple plants, leaf chlorophyll index and growth were not affected by the form of Fe (chelated or non-chelated Fe), suggesting that non-chelated Fe may be a suitable Fe source for pond apple plants in soils subjected to short-term flooding. Plants in the non-chelated, non-flooded treatments had the lowest leaf chlorophyll index and growth compared to the other Fe and flooding treatments.

An increased root inducible FCR activity by Fe deficiency is one of the most common physiological responses of Strategy I plants (dicots and monocots except the graminaceae). The root FCR activity of 4-months-old seedlings of pond apple and soursop grown in nutrient solution was not induced by lack of Fe. Pond apple plants grown without Fe exhibited leaf chlorosis symptoms within 3 weeks after Fe treatments were initiated. Lack of Fe in the nutrient solution resulted in a lower leaf chlorophyll

index and Fe concentration, shoot growth, and fresh and dry weights for pond apple than soursop.

The responses of pond apple and soursop to low Fe concentrations compared to an absence of Fe were also evaluated. Root FCR activity of pond apple was not induced by a low Fe concentration of 2 μM but was induced at concentrations of 45 μM Fe. In general, root FCR activity of soursop was not affected by Fe concentrations in the nutrient solution, although 2 μM Fe tended to decrease root FCR activity. The existence of leaf FCR activity in pond apple and soursop was confirmed, and that activity was reduced when a small amount of Fe (2 μM) was used in the nutrient solution. Soursop appears to be more efficient in reducing Fe^{3+} -citrate in leaf cells than pond apple since soursop exhibited higher leaf FCR activity than pond apple. Pond apple had lower leaf Fe concentrations than those reported as the sufficiency range for other *Annona* species such as atemoya growing under the same conditions as soursop. These results suggest that pond apple requires high levels of Fe to maintain its rapid growth rate. The differences between pond apple and soursop in FCR activity and growth to Fe concentration in the nutrient solution may partially explain their differences in tolerance to Fe-deficient soils. The higher leaf FCR activity and slow growth rate may explain why soursop did not exhibit severe leaf chlorosis compared to pond apple. Soursop maintained adequate stem growth even at low Fe levels, suggesting that it may tolerate low Fe conditions. The ability of soursop to tolerate low Fe conditions and its slower growth relative to pond apple may be advantageous when using soursop as a rootstock, since vigorous rootstocks may induce excessive growth of the scion and reduce yields. Pond apple had relatively low root and leaf FCR activities probably due to its native origin in wetland areas, where

there is sufficient soluble Fe in the soil for plant growth and development. Therefore, pond apple presumably did not have selection pressure for developing physiological mechanisms such as increased root FCR activity for Fe uptake and reduction that were observed in soursop. Pond apple and soursop require some Fe to induce root FCR activity since the lack of Fe in the nutrient solution did not induce root FCR activity as reported in many crops (Castle and Manthey, 1998; Nikolic et al., 2000; Marler et al., 2002). The results from these studies may not be sufficient to classify soursop as an Fe-deficiency tolerant species because these responses may be masked by plant age. Soursop plants used in these studies were 4-months or 1-year-old, and woody plants can store large amounts of Fe in their roots (Mengel, 1994).

The only two known flood-tolerant *Annona* species, soursop and pond apple require different soil temperatures and Fe nutrition for optimum growth. This information may help in utilizing these species as flood-tolerant *Annona* rootstocks adapted to a wide range of soil temperatures and Fe fertility. These results may be useful in nutrient management in alkaline soils, thus improving utilization of nutrients by pond apple and soursop, and reducing potential pollution of surface and groundwater.

APPENDIX A
ANALYSES OF VARIANCE FOR THE EFFECT OF FE FERTILIZATION AND
FLOODING ON LEAF MACRONUTRIENT CONCENTRATIONS OF POND APPLE
12 WEEKS AFTER TREATMENTS WERE INITIATED

Source of variation	Nutrient				
	N	P	K	Ca	Mg
Main factors					
Flooding	**	**	**	*	*
Iron source	**	NS	**	**	**
Iron rate	NS	*	NS	NS	NS
Interactive effects					
Flooding *Iron	**	NS	**	**	**
Flooding*Rate	NS	**	*	NS	NS
Iron*Rate	NS	NS	NS	**	*
Flooding*Iron*Rate	*	*	NS	NS	**

*, **, NS Significant F test at $P < 0.05$ and 0.01 , and non-significant, respectively

APPENDIX B
ANALYSES OF VARIANCE FOR THE EFFECT OF FE FERTILIZATION AND
FLOODING ON LEAF MICRONUTRIENT CONCENTRATIONS OF POND APPLE
12 WEEKS AFTER TREATMENTS WERE INITIATED

Source of variation	Nutrient		
	Mn	Zn	Cu
Main factors			
Flooding	**	**	**
Iron source	NS	NS	NS
Iron rate	NS	**	NS
Interactive effects			
Flooding *Iron	*	NS	*
Flooding*Rate	NS	**	NS
Iron*Rate	NS	NS	NS
Flooding*Iron*Rate	NS	NS	NS

*, **, NS Significant F test at $P < 0.05$ and 0.01 , and non-significant, respectively.

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BIOGRAPHICAL SKETCH

Maritza Gisela Ojeda Mosquera attended the University Centroccidental “Lisandro Alvarado” (UCLA), Barquisimeto, Lara, Venezuela where she received her bachelor’s degree in agricultural engineering in 1985. After graduating, she worked on agricultural development and research projects in Yaracuy and Lara states in Venezuela from 1985 through 1989. Since 1990 she worked as a faculty member in the Biology Sciences Department in the College of Agronomy at UCLA. She simultaneously attended graduate school at UCLA and in 1996 received her master’s degree in horticultural sciences, majoring in fruit crops. In 1999, she began her Ph.D. degree in physiology of fruit crops in the Horticultural Science Department at the University of Florida (UF) under the supervision of Dr. Bruce Schaffer.

While at UF, Maritza was elected to Alpha Zeta, the national agricultural honorary fraternity. She has also received awards from the Muriel Rumsey Foundation and Miami-Dade County AGRI-Council. Additionally, she has received research awards from the UCLA and FONACIT in Venezuela.

Maritza has worked continuously in research, extension and/or teaching in the areas of fruit, vegetables, and agronomic crops since 1985. Her research interests focus on production physiology and management of fruit crops.

After completing requirements for the Ph.D. program, she will return to Venezuela where she will resume employment as a faculty member in plant physiology with the UCLA.

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